Synthesis and characterisation of macromolecular carriers of methotrexate

Marriott, Robin John

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Thesis

Submitted by Robin John Marriott BPharm, MRPharmS,

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Ann Arbor, MI 48106-1346

To my parents

ABSTRACT

Methotrexate (MTX) was covalently linked to human serum albumin using the water soluble carbodiimide reagent 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide (ECDI) and also using the N-hydroxysuccinimide active ester of MTX, molar conjugation ratios of up to 26 being achieved. MTX was also linked (via ECDI) to a poly (L-lysine-L-glutamic acid) copolymer which was synthesised by the copolymerisation of the N-carboxyanhydrides of carbobenzoxy-lysine and benzyl-glutamate using diethylamine as initiator. The molecular weight of the polymer was determined from viscosity measurements on the protected copolymer and confirmed by fast protein liquid chromatography (FPLC) of the deprotected copolymer. The high molecular weight conjugates of MTX were isolated by Sephadex gel filtration chromatography. The in vitro stability of MTX-carrier (HSA or copolymer) conjugates in buffer (pH7.4 and 5.6) was determined in the presence and absence of enzymes (trypsin and tritosomes) at 37°C. The release of free MTX was monitored using an FPLC column (Superose 12 or TSK G3000 PWxl) which was also used to determine the molecular weight distributions of the samples (by analysis of the output from a UV detector using a BBC Master series microcomputer). The stability experiments showed that at pH7.4 up to 5% free drug was released from MTX-HSA conjugates over a 24 hour period. The MTX-copolymer conjugates were shown to be stable under the same conditions. Incubation with enzymes had little effect on the release of free MTX. The presence of drug conjugated to the carrier did not prevent the enzymatic degradation of the carrier unless drug loading was extremely high. It was found that both enzyme systems brought about a reduction in the molecular weight of MTX-HSA conjugates into a series of identifiable fractions. Trypsin was found to

have a higher activity at pH7.4 and resulted in an increase in the polydispersity index of the drug-carrier conjugate with fragments at high, intermediate and low molecular weight. The tritosomes, which were more active at the lower pH and were dependent on the presence of reduced glutathione, caused a large increase in polydispersity with material being either high or low molecular weight (ie MTX-oligopeptides). The MTX-L-copolymer conjugates were degraded to lower molecular weight derivatives of MTX in a similar manner to MTX-HSA systems however the molecular weight distribution of MTX-D-copolymer conjugates was unaffected by either enzyme system.

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C O N T E N T S

CHAPTER FOUR: SYNTHESIS, CHARACTERISATION AND DEGRADATION OF MTX-COPOLY(AMINO ACID) CONJUGATES

ABBREVIATIONS

A/I - anhydride/initiator ratio
ADP - adenosine diphosphate
ATP - adenosine triphosphate
Au - gold
BGA - benzyl glutamate anhydride
BSA - bovine serum albumin
CLA - carbobenzoxylysine anhydride
CURL - compartment for uncoupling receptor and ligand
CDI - carbodiimide
COP - copolymer (lysine-co-glutamate)
DMF - dimethylformamide
DNR - daunorubicin
DHFR - dihydrofolate reductase
DNA - deoxyribonucleic acid
DCC - dicyclohexyl carbodiimide
DEA - diethylamine
DP - degree of polymerisation
ECDI - 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EGF - epidermal growth factor
ER - endoplasmic reticulum
FPLC - fast protein liquid chromatography
FH_2 - dihydrofolate
FH_4 - tetrahydrofolate
GPC - gel permeation chromatography
GSH - reduced glutathione
HPMA - N(2-hydroxypropyl)methacrylamide
HSA - human serum albumin
HRP - horseradish peroxidase
LDL - low density lipoprotein
MTX - methotrexate
MSA - murine serum albumin
MW - molecular weight
MCR - molar conjugation ratio
MCA - monoclonal antibody
NAp - p-nitroaniline
NHS - N-hydroxysuccinimide
NCA - N-carboxyanhydride
NADPH - nicotinamide adenine dinucleotide phosphate
NMR - nuclear magnetic resonance spectroscopy
PLL - poly-L-lysine
PVP - polyvinyl pyrrolidone
PBS - phosphate buffered saline
RES - reticuloendothelial system
RNA - ribonucleic acid
TAA - tumour associated antigen
UV - ultraviolet (spectroscopy)

Origins and scope of this study

HSA has previously been investigated as a carrier system for MTX and has been shown to be cytotoxic to L1210 tumour cells in vitro and in vivo (Chu and Whiteley, 1979). Combination of such conjugates with a monoclonal antibody also demonstrated selective toxicity against in vitro cell lines (Garnett et al, 1983). MTX covalently linked to polyamino acids (mainly poly-L-lysine) has also been examined as a potential carrier system since it has the ability to enhance drug uptake (Shen & Ryser, 1979). The potential of such a system is limited due to the cationic nature of the polymer which results in indiscriminate cellular binding.

It was the purpose of this study to investigate the factors affecting the covalent linking of MTX to HSA and to monitor the solution stability of the conjugates so formed. It was thought important to quantify the release of free MTX since this would affect the in vitro cytotoxicity and consequently any results which have been attributed to the conjugate alone (Chu and Whiteley, 1979, Garnett et al, 1983).

Copolymers (of L-lysine- and L-glutamic acid) were synthesised for evaluation as carriers since it was proposed that the net charge on the molecule would be less than for either homopolymer. The polymer should also provide a larger number of sites for linkage of MTX than HSA, in a lower molecular weight molecule. The presence of both amine and carboxyl groups may also provide possible sites for linkage of different types of cytotoxic drugs.

The effect of enzymes (ie trypsin and tritosomes) on the release of free MTX from the conjugates was assessed along with the breakdown of the

macromolecule into different molecular weight fractions. This was in order to discover whether the MTX would be released in the free form or as oligopeptide derivatives.

The in vitro release (in buffer pH7.4 and 5.6) of the drug was determined using Fast Protein Liquid Chromatography. The data from the UV detector was also captured by an Acorn BBC Master series microcomputer/Winchester disk and a programme developed to determine the molecular weight distributions of the samples following incubation in the presence and absence of enzymes.

CHAPTER ONE: INTRODUCTION

1

1.1 Introduction to site-specific delivery of drugs

The entry of small molecules into a cell may be either by passive diffusion or active transport. In the case of cancer chemotherapy the cytotoxic drugs can circulate in the blood stream and may be taken up by normal and cancerous cells alike, leading to the occurrence of side effects.

The biodistribution and cellular uptake of drugs can be modified by increasing their molecular dimensions (eg by incorporation into liposomes (Gregoriadis et al, 1982)), incorporation into or linking to particulate carriers (eg albumin microspheres (Widder et al, 1981) or cyanoacrylate nanoparticles (Couvreur et al, 1980)) or by linking to soluble macromolecules. The increase in size restricts cellular uptake to pinocytosis (fluid phase or adsorptive) or phagocytosis, in the case of the particulate material. Particles over 50nm in diameter are unable to leave the vascular system except in organs such as the liver and spleen where the blood capillaries have a discontinuous lining of endothelial cells. The majority of particulate matter is phagocytosed by the fixed macrophages of the reticulo-endothelial system (RES), notably the Kupffer cells of the liver. The uptake of such particles is affected by their size and surface properties, consequently their fate can be influenced to some extent by the adsorption of proteins, sugars or surfactants. In the case of soluble carrier systems the macromolecules need to be sufficiently large to avoid glomerular filtration (MW c.30,000) but small enough to escape from the vascular system ie IgG (MW = 160,000) is extravasated but it is unlikely that IgM (MW = 900,000) leaves the circulation (Taylor and Granger, 1984).

Passive targeting (ie linking of a drug to a carrier) may be sufficient to increase the therapeutic index alone eg diseases of the macrophage system or RES. However active targeting may be necessary to achieve delivery to other groups of cells. This approach requires the incorporation of a more specific site directing agent which is recognised by cell surface receptors (with subsequent rapid uptake by receptor mediated pinocytosis) or that attach to cell membrane components (followed by uptake by adsorptive pinocytosis). The use of antibodies may be of particular importance since they exhibit exquisite specificity and can be raised against specific cell surface antigens.

The simplest site specific drug delivery system would thus comprise drug linked to an antibody however it has been shown that only a limited number of drug molecules can be combined without loss of antibody specificity (Ghose and Blair, 1978).

The use of a carrier system is thus implicated to increase the drug loading per antibody in order to achieve a therapeutic level intracellularly. The delivery system should be as small as possible to facilitate extravasation with a high drug loading (avoiding insolubility problems).

1.2 Cellular internalisation and fate of macromolecules

The term lysosomotropic agents was adopted by de Duve (de Duve et al, 1974) to describe substances that were selectively taken up into the lysosomes, it was further proposed that the phenomenon could also find application in the area of drug delivery. The lysosomes themselves are

cell organelles which are present in all eukaryotic cells; they are polymorphic in nature (in terms of size, shape and internal structure) and comprise a variety of acid hydrolases encapsulated in a lipoprotein membrane (de Duve, 1983). The lysosomal enzymes number in excess of 50 and comprise proteases, nucleases, glycosidases, aryl sulphatases, lipases, phospholipases and phosphatases (Barrett, 1972). The enzymes generally have acidic pH optima and are held within the acidic milieu of the lysosome (pH4 to 5), where they are capable of dismantling a wide variety of complex materials (mostly naturally occurring macromolecules eg. proteins and polypeptides). The integrity of the membrane is important since it prevents cellular autolysis by the powerful enzymes although the dilution effect coupled with the elevated pH of the cytosol would render them less active. Weak bases (eg dyes and chloroquin) are examples of lysosomotropic (or acidotropic) agents which become trapped within the lysosomes by the pH-partition effect whereby protonation of the base renders it incapable of crossing the cell membrane. A further example is the surfactant Triton WR1339 which also accumulates in the lysosome, a phenomenon which is employed in order to isolate modified lysosomes since it reduces their density (Trouet, 1974).

The entry of substances into the cell may be either by permeation or endocytosis. In the former case molecules (depending on their molecular size and charge) enter the cell by random diffusion unless an active transport mechanism exists (eg. folate transporter).

Endocytosis is the process of internalisation of formerly extracellular material brought about by the invagination of the plasma membrane followed

by the formation of a bound vesicle containing extracellular fluid. The term encompasses both phagocytosis and pinocytosis. Phagocytosis occurs in specialised phagocytic cells eg macrophages. This is the uptake of particulate matter (generally > 1um in diameter, eg latex beads) and is triggered by the interaction between the particle and the plasma membrane causing the particle to be engulfed.

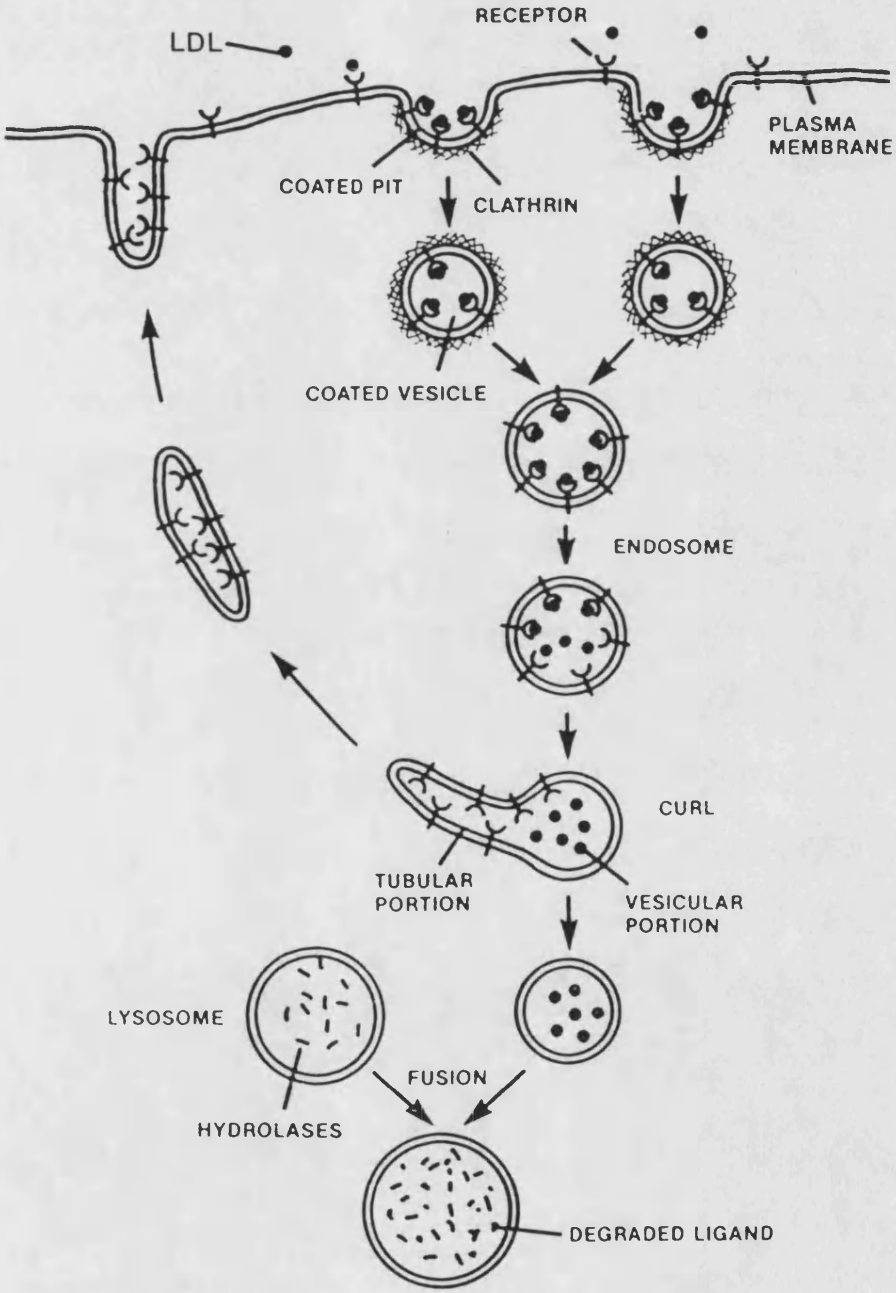
Pinocytosis occurs in nearly all cells and may be fluid phase or adsorptive in nature, the latter may be further subdivided into selective and non-selective mechanisms. Fluid phase pinocytosis is the entrainment of solutes (or small particulates up to 50nm in diameter) within fluid droplets when the membrane vacuolises to form a pinosome. In the case of adsorptive pinocytosis the substrate (eg macromolecules) binds to the plasma membrane and is internalised with the pinosome (Lloyd and Williams, 1984) this results in an increased rate of uptake over the fluid phase mediated process (Duncan et al 1984).

Some molecules have specific cell surface receptors and enter the cell via receptor mediated (selective) pinocytosis whereby the ligand eg. low density lipoprotein, hormones (EGF) or plasma transport proteins (trans-cobalamin) occupies a receptor resulting in internalisation of the receptor-ligand complex (Figure 1.1). The receptors (transmembrane proteins) when occupied by ligands tend to cluster into special membrane areas that are invaginated forming coated pits. The clustering process may occur by the complex encountering a coated pit whilst randomly diffusing through the lipid bilayer (Schlessinger et al, 1978) since the coated pits occupy about 2% of the cell surface. The main component of the coating of the coated pit is the protein clathrin (Pearse, 1976) which is composed of

a heavy chain (MW = 180,000) and several light chains (MW 20-40,000). Clathrin in its purified form is a three-armed trimer of three heavy and three light chains called a triskelion. These units have the ability to polymerise and form a cage like structure which may cause the pit to expand and pinch off from the membrane (Crowther and Pearse, 1981). It is thus possible that all pinosomes are formed from coated pits.

The invaginations eventually form discrete coated vesicles which rapidly lose their clathrin coat (15-60 seconds) (Helenius et al, 1983) and may fuse to form larger vesicles (endosomes) (Brown et al, 1983). The pH within the endosome has been shown, using fluorescein labelled α-2-macroglobulin, to be acidic with the pH dropping to 5-6 after about 15-20 mins (Tycko and Maxfield, 1982). The drop in pH causes the receptor-ligand complex to separate with the receptors tending to accumulate in a tubular extension, which has a high surface to volume ratio, from the vesicle (called the CURL; compartment of uncoupling of receptor and ligand). It is proposed that the tubular portion then splits off and returns to the plasma membrane whilst the vesicular portion may either fuse with a lysosome forming a secondary lysosome or possibly be exocytosed. Evidence for the recycling of receptors has been supplied by the fact that ligand internalisation continued for several hours after receptor synthesis had been blocked; also raising the endosomal pH with NH_4Cl (brought about a depletion of surface receptors (Goldstein and Brown, 1974). In some cases ligand binding initiates clustering (eg EGF) and in others ie LDL the coated pits/LDL receptors are continuously being recycled (Anderson et al, 1982). It is likely that much of the plasma membrane is recycled along with the receptors since it has been calculated that endocytosis accounts for up to 200% of the cell surface per hour.

Figure 1.1 Diagram of receptor-mediated pinocytosis and subsequent fate of low density lipoprotein



The reduced pH within cellular organelles ie. endosomes and lysosomes is thought to be due to an active proton pump as opposed to the buffer capacity of the vesicular fluid and it has been shown that the pH lowering capacity is dependant on the presence of ATP (Harikumar and Reeves, 1984). The acidity within the endosome/lysosome is necessary to achieve receptor-ligand decoupling and also for some of the enzymes to be active but may also cause accumulation of certain substances eg weak bases as previously mentioned. In the case of transferrin there is a rapid drop to pH 6 followed by a slow decrease to pH 5. The endosomal pH increases during recycling (Corley Cain et al, 1989).

The lysosomal membrane is impermeable to macromolecules hence accumulation could occur if the molecule cannot be degraded by the enzymes. If the macromolecule is degraded (as is likely in the case of peptides) then only low molecular weight molecules, eg. amino acids and dipeptides, can cross the membrane either by diffusion or possibly an active carrier (Forster and Lloyd, 1988). The degradation products may thus accumulate in the secondary lysosome, be released into the cytosol or be exocytosed.

It is also proposed that the Golgi apparatus may play a part in the cellular movement of endosomes and lysosomes (Pastan and Willingham, 1983). It is postulated following studies on transferrin and alpha-2-macroglobulin, that the receptosomes may fuse with elements of the sorting system eg. the trans-reticular elements of the Golgi system which rapidly transfers ligands into lysosomes (Goldenthal et al, 1988).

1.3 Macromolecular carriers for site-specific delivery of drugs

1.3.1. Introduction

The use of many antineoplastic agents has been restricted by their lack of ability to discriminate between normal and transformed proliferating cells. In an attempt to acquire some site specificity many workers have used carrier molecules to transport drugs to target tissues. An effective system has the potential to dramatically improve cancer chemotherapy since one of the most serious problems with such therapy is the occurrence of toxic side effects (Arnold et al, 1983). The requirements of a (lysosomotropic) carrier are as follows (de Duve et al, 1974, Ghose and Blair, 1978):

i) the binding of the drug to the carrier must be unaffected by body fluids but be reversible by lysosomal enzymes yielding an active compound.

ii) the conjugate must exhibit specificity for the target site and reach it in vivo.

iii) the conjugate must be internalised by the cell (charge, molecular size and functional groups present may be important to stimulate pinocytosis).

iv) the carrier should be non-toxic (although preferential toxicity for the target site may be beneficial).

v) the conjugate must lack immunogenicity and must not exhibit non-specific binding to molecules of the immune system (the formation of immune complexes may lead to non-specific uptake particularly by the liver and spleen; immunogenic conjugates would lead to hypersensitivity reactions on repeated administration).

vi) the number of drug molecules per carrier molecule must be high enough to achieve cytotoxic concentrations within the target cell.

Several different carriers linked to a variety of compounds have been used *in vitro* and *in vivo* eg.:

Carrier	Drug	Reference
Human serum albumin	MTX	Chu and Howell, 1982
Bovine serum albumin	MTX	Chu and Whiteley, 1977
Murine serum albumin	MTX	Chu and Whiteley, 1977
Poly (L-Lysine)	MTX	Shen and Ryser, 1979
Poly (D-Lysine)	MTX	Shen and Ryser, 1979
Poly (ethyleneimine)	MTX	Fung et al, 1979
HPMA	Daunomycin	Duncan et al, 1987
HPMA	Puromycin	Duncan et al, 1987
DNA	Daunorubicin	Trouet et al, 1982

(HPMA = N-(2-hydroxypropyl)methacrylamide)

In addition to the above carriers the following have also been used: hormones (which possess a high degree of specificity for target cells (Varga et al, 1977)), lectins (Yamaguchi et al, 1979), liposomes (Gregoriadis, 1982) and antibodies (Ghose et al, 1976).

1.3.2 Albumin as a carrier

Chu and Whiteley (1977) proposed that by synthesizing a higher molecular weight derivative of MTX, by linking it to bovine serum albumin (BSA) or murine serum albumin (MSA), a prodrug could be produced which would retain the properties of the carrier summarised below:

i) the conjugate would be retained within the body forming a depot of MTX which would be slowly hydrolysed maintaining elevated blood levels.

ii) if the conjugate had sufficient molecular weight it may be restricted to a body cavity containing a solid tumour.

These authors found that MTX remained covalently bound to the albumin carrier in the plasma.

MTX was coupled to BSA, MSA (and dextran) by condensing a terminal carboxyl group of the antifolate molecule to reactive ϵ-amino groups in lysine residues of the albumins. The reaction was promoted by carbodiimide reagents (Mell et al, 1968) and UV analysis was used to estimate that an average of 12 moles MTX were linked per mole of albumin.

L1210 tumour bearing mice treated with the conjugate were used to demonstrate that a higher more prolonged extracellular concentration of drug could be achieved when compared to that achieved using free MTX. The authors suggested that MTX-albumin conjugates were retained in the serum until hydrolysed (90% of free MTX was found to be intracellular). The conjugate was found to be superior to free MTX against Lewis Lung carcinoma in mice (Chu and Whiteley, 1979).

Chu and Whiteley (1979) showed that MTX was not hydrolysed from a BSA carrier at the plasma membrane (evidenced by low serum levels of MTX) Radiolabelling experiments showed that hydrolysis occurred within the cell and using inhibitors of MTX transport it was shown that carrier-bound drug was transported into the cell by a different mechanism to that used by the parent compound. From studies using Dextran T70 (which showed no cellular binding and low drug binding) the authors suggested that the capacity to bind to a membrane surface was one requirement of a carrier and concluded that proteins may be useful as carriers for MTX.

The use of HSA as a drug carrier system has been further explored by Trouet et al (1982); in this case daunorubicin (DNR) was linked to a succinylated derivative of BSA using amino acid spacers (0-4 peptides) producing molecules of the type shown below:

Albumin-NH-CO$(CH_2)_2$-CO-Ala-Leu-Ala-Leu-DNR

The linkage of DNR or one of its derivatives to succinylated BSA was via the free amino group on the drug and the succinyl carboxylic acid residue. Linkage was achieved using a carbodiimide reagent. Incubation of the

conjugates with rat liver tritosomes revealed that in the case of the tetrapeptide 75% of the bound DNR was released as the free drug after 10 hours. After a similar period only 60% was released from the tripeptide and only 8% from the dipeptide. When tested against mice which previously had been injected intraperitoneally with L1210 cells, the chemotherapeutic effect followed a similar trend to that noted for the DNR release; the tetrapeptide being the most effective. It was also shown that the conjugates were less toxic than the free drug. Trouet et al (1982) concluded that it was necessary to insert a tri- or tetrapeptide to enable release of free drug in the lysosomal environment which was capable of exerting its cytotoxicity. It was proposed that this peptide spacer could be used to facilitate release of DNR from any suitable carrier molecule.

1.3.3 Polylysine as a carrier

Polylysine itself has been shown to have cytotoxic properties (Arnold et al, 1979) against Hela cells in culture and Ehrlich ascites carcinoma in white Swiss mice. The effects were shown to be dependent on both molecular weight (increase in MW led to increased cytotoxicity) and dose. No antineoplastic activity was evident with L1210 leukaemia in mice. The cytotoxicity against the Hela cells probably resulted from direct interaction between the cationic polylysine and the overall electronegative charge of cell surfaces (Pitha, 1983). The charge arises due to glycoproteins present in the plasma membrane and many types of malignant cells have been shown to have higher surface negative charge than normal cells (Smets, 1980). The cytotoxicity may arise due to an

increase in the permeability of the membrane due to polycationic interaction with electronegative regions causing openings in the lipid bilayers as has been shown for erythrocyte membranes (Katchalsky, 1964). The in vivo cytotoxicity against Ehrlich ascites carcinoma was probably also due to direct interaction between polymer and cell, although the possibility exists that the immune response of the animal was stimulated. The effects of poly (L-lysine) (PLL) binding to the cells are as follows: rapid leakage of small molecules/ions across the cell membrane (Kornguth and Stahman, 1961) followed by rapid loss of RNA and DNA resulting in loss of protein synthesis leading to cytotoxicity.

Ryser and Hancock (1965) showed that uptake of protein molecules (eg albumin) was enhanced by low concentrations of basic polyamino acids. In further studies by Ryser (1967) it was found that increasing the molecular weight of poly-L-lysine increased the uptake of albumin by S180 cells (200 fold increase over the range 19,000-1,130,000 Daltons). Poly-D-lysine proved to be more effective than poly-L-lysine at promoting uptake. DEAE-Dextran (diethylaminoethyl-dextran), another basic polymer, was also shown to increase albumin uptake and it was concluded that attachment of a large basic polymer to membrane constituents and the conformational changes resulting from this interaction stimulated pinocytosis. Pratten et al (1978) and Duncan et al (1979) investigated the stimulation of pinocytosis using polyamino acids (of lysine, ornithine and glutamic acid) and dextrans using ^{125}I-labelled PVP as a fluid phase marker. The adjuvants had little effect on the rate of uptake of radioactivity by either rat peritoneal macrophages or the rat yolk visceral sac. Further experiments using ^{198}Au(colloidal) and ^{125}I-PVP showed enhanced uptake of ^{198}Au in the presence of polycations (PLL, poly-D-lysine and

poly-L-ornithine) by rat macrophages but not by the yolk sac. The enhanced uptake was thought to be due to aggregation of the gold and increased affinity of the polycation-colloidal gold complex for the plasma membrane leading to endocytosis of the complex, although this was not the case for all cells. The uptake of ^{125}I-PVP was relatively unaffected dismissing the theory that increased pinocytosis can be caused by polycations.

Ryser et al (1978) showed that the membrane transport of the enzyme horseradish peroxidase was increased by 1000 fold (over 60 minutes in L929 mouse fibroblasts) by covalently attaching it to PLL (MW 6,700). The fact that PLL was an effective transport vector for molecules larger than itself led these authors to investigate conjugates with smaller molecules; namely MTX (Ryser and Shen, 1978). MTX was linked to PLL (average MW 70,000) in a carbodiimide-mediated reaction to produce conjugates of molar ratios 13:1 (MTX:PLL). The conjugate was shown to be an effective toxin against cultured Chinese hamster ovary cells which were MTX-resistant due to deficient MTX transport. This suggested a different mechanism of cell entry (ie. piggyback endocytosis). The conjugate failed to bind to DHFR in vitro; this may have been due to charge density and not molecular size since polyglutamates still bind DHFR. It has also been shown that a MTX/PLL/heparin complex bound to DHFR in vitro (Shen and Ryser, 1981a) due to charge reduction by heparin. These data suggested that intracellular hydrolysis must have occurred after transport of the MTX into the cell. If brief trypsin digestion of the MTX-PLL preceded the cell culture experiments enhancement was abolished; the same was shown for horseradish peroxidase/PLL conjugate (Ryser et al, 1978). These experiments supported the view that MTX-PLL entered the cell by adsorptive

pinocytosis. The fact that intact conjugates were internalised by a route other than by the active MTX transporter was confirmed using thiamine pyrophosphate which inhibited MTX transport in L1210 cells but had no effect on uptake of MTX-PLL (Shen and Ryser, 1981b). Leucovorin however protected cells from both MTX and MTX-PLL since it does not affect transport mechanisms but alters the intracellular folate pool (Jolivet et al, 1983). Intracellular degradation of the internalised MTX-PLL conjugate was thought to yield either MTX, MTX-lysyl or MTX oligolysyl peptides or a combination thereof (this was inferred due to the lack of MTX-PLL DHFR binding in vitro).

MTX coupled to poly-D-lysine (MW 60,000) (Shen and Ryser, 1979) showed no inhibitory effect on drug resistant Chinese hamster ovary cells since it would not have been broken down by lysosomal enzymes, though internalisation presumably occurred at a similar rate to that of MTX-PLL. Horseradish peroxidase studies (Ryser et al, 1978) showed that HRP-PLL conjugates were present in endosomes. It seems likely that MTX-PLL conjugates follow the same route and are digested within secondary lysosomes allowing release of MTX (and possibly lysyl derivatives) into the target cell.

The idea of confining a conjugate to a body compartment was investigated using mice containing L1210 tumour treated with MTX-BSA and MTX-PLL (Chu and Howell, 1981). Leucovorin was employed to block the toxicity of any escaping MTX. MTX-BSA was retained for 48 hours in contrast to MTX-PLL which was cleared within 4 hours. This may have be due to the positive charge on the PLL at physiological pH and its subsequent affinity for cell surfaces, leading to increased cellular uptake and decreased intracavity

half-life (PLL of MW 40,000-60,000 being cleared more rapidly than PLL of MW 3,000). The half-life for MTX was increased by linking to BSA but its cellular uptake was poor and blood clearance slow.

Galivan et al (1982) showed, using tritiated MTX, that uptake of MTX-PLL was equivalent in both resistant and non-resistant (re. MTX transport) hepatoma cells in vitro. MTX-PLL was found to be much more effective at inhibiting growth than PLL (MW 35,000) alone (Whiteley et al, 1981) whereas the coupling of MTX to poly-D-lysine had no effect on growth. Sephadex gel filtration of cell extracts showed that MTX-Poly-D-lysine remained conjugated whereas MTX-PLL had been hydrolysed yielding free MTX and some higher molecular weight derivatives; either polyglutamates or oligolysyl derivatives. DHFR also exhibited radioactivity due to the presence of bound ^{3}H-labelled MTX. Leupeptin, an inhibitor of lysosomal cathepsins (thiol proteases) in hepatic cells (Seglen et al, 1979), prevented the liberation of MTX from PLL indicating that cleavage does in fact occur by a lysosomal mechanism. The hydrolysis of MTX-PLL was reduced by the addition of NH_4Cl to (another lysosomal inhibitor (Seglen et al, 1979)) to the incubation medium again indicating a lysosomal mechanism. Folinic acid (leucovorin) blocked the uptake of MTX but not MTX-PLL in H35 non-resistant cells indicating that endocytosis of MTX-PLL was the mechanism of its internalisation.

1.3.4 HPMA as a carrier

A model synthetic (biodegradable) macromolecule has been investigated by Kopecek and coworkers (1982) based on a non-degradable polymer backbone ie

N(2-hydroxypropyl)methacrylamide (HPMA). Biodegradability was introduced into the system by way of oligopeptides which were used to both cross link the polymer chains and also as spacers to incorporate model drugs and vectoring agents into the carrier system. The composition (number, type and sequence) of these oligopeptides were altered in order to investigate the enzymatic degradation of the polymer and release of model drug p-nitrophenol. The peptides could be varied to suit enzymes with known specificities (eg chymotrypsin and trypsin) and it was found that a tetrapeptide spacer (minimum) was necessary to allow maximal cleavage, presumably due to steric problems (and enzyme specificity). The tritosomal degradation of a variety of conjugates were also investigated (Duncan and Lloyd, 1980) with the result that release of model drug occurred; the sequence could thus be tailored either to yield a particular release rate or to a particular enzyme within the lysosomes. The systems were found to be essentially stable in rat plasma/serum (Duncan and Lloyd, 1984). It was concluded that this approach could enable the mechanism of conjugate action to be discovered and employed to improve their efficiency. Cathepsin B was found to be more active against a Gly-Phe-Leu-Gly-NAp sequence than cathepsin L or H but not as active as total tritosomes (Kopecek, 1984). The cleavage of the cross links in the HPMA polymer by tritosomes was investigated (Subr et al, 1986) and concluded that the extent of cleavage was dependent on the length and composition of the cross linking oligopeptide.

Duncan et al (1979) also demonstrated that the polymers were internalised by fluid phase pinocytosis and that the rate of uptake could be increased by incorporation of hydrophobic residues or a positive change. The oligopeptides were subsequently degraded by lysosomal enzymes (primarily

the proteases) following internalisation (Duncan and Lloyd, 1981). The linkage of HPMA to rat immunoglobulin (IgG) resulted in an increased rate, when compared to unmodified HPMA, of pinocytic uptake by the rat visceral yolk sac indicating that the antibody could still interact with membrane binding sites and had potential as a targeting moiety (Duncan et al, 1986a). The biodistribution of the delivery system was modified by incorporation of galactosamine terminated oligopeptide side chains, this resulted in rapid uptake by the liver as compared to the parent macromolecule and glucose and mannose terminated conjugates (Duncan et al, 1983). The blood clearance and liver association of the galactosaminated HPMA was found not to increase significantly when the galactosamine loading was increased above 4 mol%. It was proposed that the HPMA was degraded intralysosomally to release low molecular weight material into the bulk phase since following injection of radiolabelled HPMA into rats, material was detected in isolated liver lysosomes (Duncan et al, 1986b) . This effect was thought to be due to the presence of galactose receptors on the hepatocyte membranes (Hudgin et al, 1974).

The immunogenicity of the polymers was investigated by determining their ability to induce antibody formation in mice (Rihova et al, 1984) the polymer itself (HPMA) was non-immunogenic however attachment of the oligopeptide side chains provoked a weakly immunogenic reaction.

Daunomycin has been linked to HPMA via a peptide spacer (Gly-Phe-Leu-Gly tetrapeptide giving the maximum release rate upon incubation with tritosomes) and exhibited reduced toxicity <u>in vitro</u> against leukaemia cells when compared to free drug (Duncan et al, 1987). <u>In vivo</u> results however demonstrated an increased survival time, over the free drug, of

mice injected with L1210 cells whilst the conjugate with a non-degradable linkage was not significantly different from the untreated controls. This would infer that the conjugation resulted in reduced cardiotoxicity (Duncan et al, 1988) possibly by altering the pharmacokinetic disposition of the drug.

1.3.5 Antibodies as carriers

Antibodies are serum globulins with a wide range of specificities for different antigens. The potential use of antibodies as drug carriers for site directed therapy was originally suggested by Ehrlich who envisaged that specific antibodies could be produced against antigens.

The specificity of an antibody and its ability to combine with a particular antigen is due to the combining site of the Fab portion which is a cleft formed in the hypervariable regions of the H and L chains. The closer the fit between the combining site and the determinant (a given portion) of the antigen, the stronger the binding forces and the higher the affinity. Antigen-antibody combining forces are non-covalent (ie Van der Waals, electrostatic and hydrophobic interactions) (Roitt, 1980).

The IgG antibodies, and better still their Fab/ or F(ab/)$_2$ portions, have the greatest potential due to their low molecular weight (IgG MW is approximately 160,000 as opposed to IgM 900,000 (Roitt, 1980) allowing extravascular movement) and their abundance. Polyclonal antibodies have shown some use as drug carriers (Ghose and Blair, 1978) but they lack selectivity. The discovery of tumour-associated cell surface antigens has

permitted the production of specific antibodies by injection of human tumour cells into an animal. Tumour-associated antigens (TAA) arise during neoplastic transformation and are not detectable on normal untransformed tissue. Examples of TAAs are carcinoembryonic antigen, alphafetoprotein, ectopic hormones as well as other embryonic markers and non-foetal TAA's (Ghose and Blair, 1978). Cell surface markers (glycoproteins etc) are recognised as foreign and act as antigens leading to the production of antibodies. Obtaining an antibody specific for one antigen in useable quantities was nearly impossible until 1975 due to difficulties in purification and the quantities produced (Obrist, 1983).

Methods for production of monoclonal antibodies (MCA's) discovered by Kohler and Milstein in 1975 has solved this problem by allowing the synthesis of potentially unlimited amounts of specific MCA. All the molecules of a MCA sample are identical in amino acid sequence and hence in binding properties; the antibody can thus have exceptional specificity (Edwards, 1981).

The binding of drugs to antibodies may be achieved by non-covalent or more commonly by covalent bonds mediated by a variety of reagents eg. glutaraldehyde (Hurwitz et al, 1975), carbodiimides and activated ester intermediate (Kulkarni et al, 1981). The problems arising from coupling reactions which employ bifunctional reagents are that cross linking and polymerisation of the antibody may occur (due to the large number of functional groups present) resulting in a loss in specificity. A loss of antibody activity may also result from drug substitution at the antigen binding site (Gros et al, 1981) or alteration in the tertiary structure of the antibody arising from multiple substitution. The following are

examples of cytotoxic agents which have been linked to immunoglobulins.

1.3.5.1 Alkylating agents

Chlorambucil (an aromatic derivative of Nitrogen mustard) has been linked to antitumour globulins (by carbodiimide) and has been successfully employed in the treatment of tumour inoculated mice (Tai et al, 1979).

1.3.5.2 Intercalating agents

Hurwitz (1983) linked daunomycin either directly to antibody or via a dextran bridge. In all cases a drop in antibody activity was observed. The reduction was greater when the dextran spacer was incorporated (50% activity retained). The spacer also caused a drop in pharmacological activity but allowed an increased level of substitution over direct linking of drug to the antibody. It was found from in vivo experiments that linking the drug to a macromolecular carrier brought about a reduction in the toxic side effects, that specific carriers were more effective than non-specific carriers in several tumour systems and that both were an improvement over the free drug. In contrast to this Aboud-Pirak et al (1989) showed that daunorubicin linked (via a tetrapeptide spacer) to a monoclonal antibody was inactive even though antibody activity was retained and drug was released in the presence of lysosomal enzymes in vitro.

1.3.5.3 Antimetabolites

Initially MTX was linked to antitumour immunoglobulin using a diazo reaction and employed successfully to treat inoculated L1210 leukaemia in mice (Mathe et al, 1958). Other attempts at diazo-mediated coupling gave variable results and often extensive precipitation (De Carvalo et al, 1964). Robinson et al (1973) demonstrated that diazotisation led to a variety of side reactions and concluded that carbodiimide-mediated linking gave a superior product exhibiting much better survival rates in animal experiments than unconjugated drug/antibody. The link produced using the carbodiimide reagent is between the amino groups of the protein and one of the carboxylic acid residues on the MTX, this is important since this does not interfere with the pteridine moiety of MTX, which is involved in binding DHFR (Bertino, 1963).

Kulkarni et al (1981) linked MTX to immunoglobulin using three different methods:-

a) water soluble carbodiimide
b) activated ester (N-hydroxysuccinimide (NHS))
c) mixed anhydride

It was concluded that the NHS method gave the best results since a conjugate with an MCR of 10 retained 90% of the antibody activity with 70% of the protein being recovered. The carbodiimide-mediated product with an MCR of 8 yielded values of 50% in both cases, however aggregation and changes in the tertiary structure were thought to be more pronounced. It was found that even with high levels of anhydride an MCR of only 2-3 could

be achieved, although 100% of antibody activity was retained. The ability of the drug to inhibit dihydrofolate reductase was found to be reduced in the case of a) and b) but abolished in the case of c). The in vivo results showed that MTX conjugated to immunoglobulin showed a significant improvement over free drug. Characterisation of synthetic methods were confirmed in a later series of experiments reported by Ghosh et al (1989) who also demonstrated that the rate of release of MTX (by hydrolysis) was dependent on pH and all conjugates, independent of the method of synthesis, had a similar pH profile showing minima at pH 7. These data indicated that the bond formed was similar in each case. Endo et al (1987a) demonstrated that MTX linked to MCA (via active ester) exhibited increased selectivity over normal murine immunoglobulin and that lysosomal degradation was in fact responsible for the activity of the conjugate. MTX linked to various MCAs showed an increased rate of tumour cell inhibition over free MTX (Smyth et al, 1987). The authors conclude that the conjugates bound specifically to their antigens/receptors (non-specific pinocytosis was minimal). Internalisation was followed by intralysosomal degradation of the conjugate. The mechanism was established by use of MTX transport inhibitors, low temperature and lysosomal inhibitors.

The MCR has been increased by the use of a copolymer intermediate (glutamic acid:lysine 4.7:1) where 63-76 moles of MTX were bound per mole of immunoglobulin (via carbodiimide) with 80-100% of pharmacological activity (inhibition of [^{3}H]-deoxyuridine incorporation) and 50-80% of antibody activity being retained (Hurwitz, 1983).

MTX has also been linked to immunoglobulins via an HSA carrier (Garnett et

al, 1983). Carbodiimide was used to obtain a conjugate with MCR of 32 MTX/HSA; 1-3 moles of MTX-HSA conjugate were then linked to one mole of monoclonal antibody (791T/36) yielding an overall MCR of 32-96. The activity of the antibody (as determined by cell cytofluorimetry) was found to be reduced to 28% of the unmodified antibody. However the conjugate exhibited increased cytotoxicity over MTX with antibody-reactive cell lines; this effect was diminished by incubation with excess antibody. This conjugate was also shown to be more cytotoxic than MTX linked directly to the antibody (via active ester) probably due to increased drug loading (Baldwin et al, 1987). Selectivity of the conjugate was demonstrated since it showed reduced activity with non-reactive cell lines. Incubation of conjugate with reactive cell lines with the incorporation of a lysosomal inhibitor (ammonium chloride) brought about a significant reduction in cytotoxicity inferring that cellular toxicity was mediated by lysosomal degradation (Garnett et al, 1984). The results of experiments with inhibitors of MTX transport lead the authors to the conclusion that free MTX released by lysosomal enzymes was transported into the cytoplasm by a specific transport mechanism. The lysosomal involvement was further supported by the *in vitro* work of Endo et al (1987b) using a different cell line.

1.3.5.4 Natural toxins

A lot of interest in the area of immunoconjugates has been concentrated on highly potent toxic glycoproteins eg diphtheria toxin (Thorpe et al, 1978), ricin (Krollick et al, 1983) and abrin (Olsnes et al, 1976). These toxins comprise two polypeptide chains (A and B, disulphide bridged, each

about 30kD) neither of which separately is toxic to cells (Olsnes, 1981). The B chain is concerned with binding to the cell surface and internalisation of the A-chain into the cytosol where it terminates protein synthesis (Thorpe and Ross, 1982). Diphtheria toxin is enzymatic in its mode of action (Collier and Kaplan, 1984) catalysing the transfer of adenosine diphosphate ribose (a part of the electron carrier nicotinamide adenine dinucleotide) to a protein called elongation factor 2. The latter is required for synthesis of protein on ribosomes and is inactivated by attachment of the ADP ribose group.

Abrin and ricin are glycoproteins which have similar structures and mechanisms of action. The B chains bind to any carbohydrate moiety on cell-surface glycoproteins or glycolipids which terminate in non-reducing galactose. There are 10^6 such receptors on human erythrocytes and up to $3x10^7$ on Hela cells (Sandvig et al, 1976) although only a few cells are able to mediate penetration of the isolated A chains through the cell membrane. The binding can be competitively antagonised by galactose or lactose thus reducing toxicity. The mechanism of entry of the A chain into the cell is unknown, it may involve endocytosis, channel formation in the membrane or may be a carbohydrate triggered mechanism (Thorpe et al, 1982). The A chains of ricin and abrin inhibit protein synthesis by inactivating the 60S subunit of the ribosome.

The toxins (or A chains) have been conjugated to either complete antibodies (Blythman et al, 1981) or Fab monomers or $F(ab)_2$ dimers (Ross et al, 1980). It is safer using the A chain alone since the complete molecule may still retain some non-specific action. However some antibodies have been conjugated in such a way as to sterically block the

receptor on the B chain (Foxwell, 1984). Those conjugates still retaining non-specific activity can be removed by binding to a column containing galactose residues.

The cell-specific cytotoxicity of these conjugates in vitro has been impressive, however in vivo results have not lived up to these promising results (Thorpe et al, 1982). One possible use of immunotoxins is in bone marrow transplantation to eradicate T lymphocytes thereby overcoming graft-versus-host disease.

Toxin A-chains have also been attached to other carriers to increase specificity eg. lectins (Yamaguchi et al, 1979), hormones (Miskimins and Schinizu, 1979) and epidermal growth factor (Cawley et al, 1980).

The aim in cancer chemotherapy is to deliver antineoplastic agents selectively to local tumour and more particularly to metastases (Baldwin and Pimm, 1983). The advent of monoclonal antibodies has been an important step toward achieving this goal providing specific binding to tumour associated antigens. The use of radiolabelled antibodies and MCA's (using ^{123}I, ^{125}I, ^{131}I and ^{111}In) has demonstrated their ability to localise in neoplastic tissue (using γ-scintigraphy coupled with computer manipulation) in induced tumours in animals (Epenetos et al, 1982). Detection of tumours as small as 1 mm in diameter has been achieved. However uptake ratios are not 100% leading to accumulation in other tissues eg. non-specific accumulation in the liver and the blood (possibly due to binding to secreted antigen). Antibody guided irradiation could also be useful therapeutically using β-emitting isotopes (Hammersmith Oncology Group, 1984) the radiation from which can penetrate about 2mm

into tissues causing cell cytotoxicity. Tumour heterogeneity can cause problems with localisation (Neville et al, 1984) as morphologically identical cells can contain different epitopes. The use of β-emitters may overcome some problems of heterogeneity but at present the efficiencies of antibody localisation are insufficient to make this a viable approach.

The use of immunotoxins is interesting since they are potentially very powerful therapeutic agents (one molecule is said to be sufficient to kill a cell). This is useful with regard to the paucity of tumour associated antigens (Kishida, 1983). Possible problems with immunotoxins are that combination with secreted antigen present in circulating plasma could lead to complexes which may be taken up non-specifically by the reticuloendothelial system and uptake by normal cells by pinocytosis. Their potency may thus lead to toxic effects on normal cells. Immunogenicity is also likely to be a problem if repeated dosing is anticipated.

The use of more conventional drugs linked to monoclonal antibodies will accentuate the problems associated with the kinetics of internalisation. The possibility exists that antibody conjugates which are attached to the cell membrane may not be internalised, therefore the use of a carrier to increase drug loading may be essential for adequate therapy. The problem of tumour heterogeneity in drug targeting may possibly be overcome by the use of a cocktail of MCAs against a variety of tumour associated antigens. Non-specific antibody interactions (ie Fc receptors on cells and the fact that Fc portions bind complement) can possibly be obviated by using Fab fragments, which may also be useful in lowering the MW of the conjugate.

Polycations may prove to be useful carriers since they have been shown to promote internalisation, however due to the electronegativity of cell surfaces non-specific binding may occur. The advantage of using a conventional drug (such as MTX) over immunotoxins is that side effects of free drug are already tolerated in the clinic thus any degree of site-specificity will lead to an improvement in chemotherapy.

1.4 Methotrexate (MTX)

1.4.1 Clinical Use

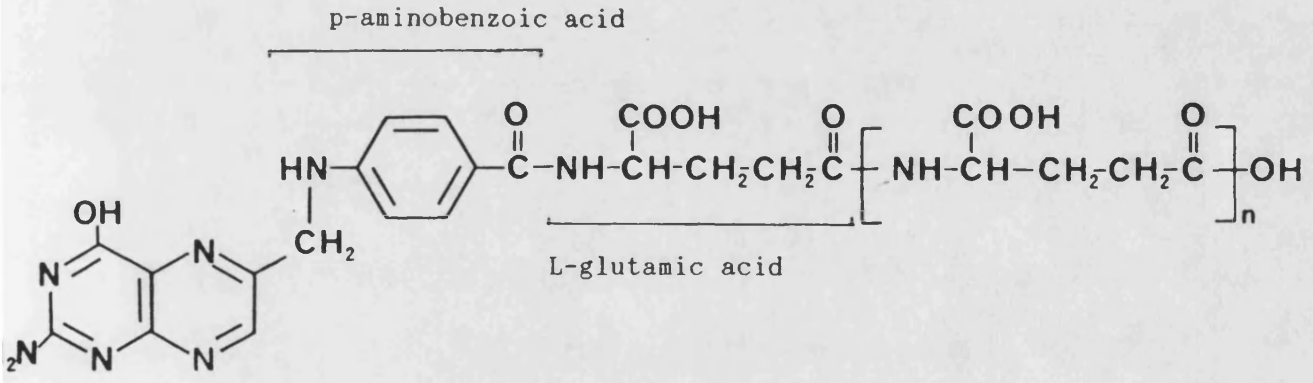
The development and use of folic acid antagonists began only a few years after the isolation and synthesis of folic acid itself; aminopterin being one of the first used for the treatment of acute lymphoblastic leukaemia in 1948 (Farber et al, 1948). Since then methotrexate has become one of the most commonly used antineoplastic folate antagonists in the treatment of a variety of malignant conditions (Table 1.1).

Table 1.1 Methotrexate-Sensitive Neoplasms

acute lymphocytic leukaemia
acute non-lymphocytic leukaemia
diffuse histiocytic lymphoma
breast carcinoma
ovarian cancer
choriocarcinoma
osteosarcoma
head and neck carcinomas
small-cell lung carcinoma
medulloblastoma

1.4.2 Biochemical Pharmacology

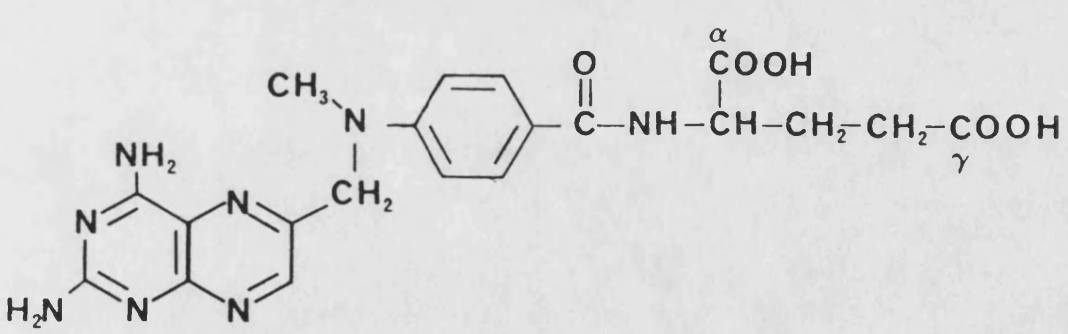
The folate vitamins are a class of essential cofactors which carry one-carbon groups required for the purine and thymidylic acid synthesis necessary for DNA production and cell division. Interference with this pathway by methotrexate has been exploited as a method of preventing cellular proliferation. Circulating folates contain a single terminal glutamate residue, however within the cell they are converted to a polyglutamated derivative by folate polyglutamate synthetase (Covey, 1980). The glutamate units are linked by peptide bonds and are retained within the cell longer than the parent compound.



polyglutamated folic acid

In order to participate in enzymatic reactions leading to purine synthesis the cofactors must be reduced to their tetrahydro form (in the pteridine ring i.e. FH_4); dihydrofolate reductase is responsible for this conversion.

Replacement of the 4-OH group with an amino moiety results in a several thousand fold increase in affinity for DHFR, MTX is a further development and is a potent inhibitor of DHFR (Werkheiser, 1963).

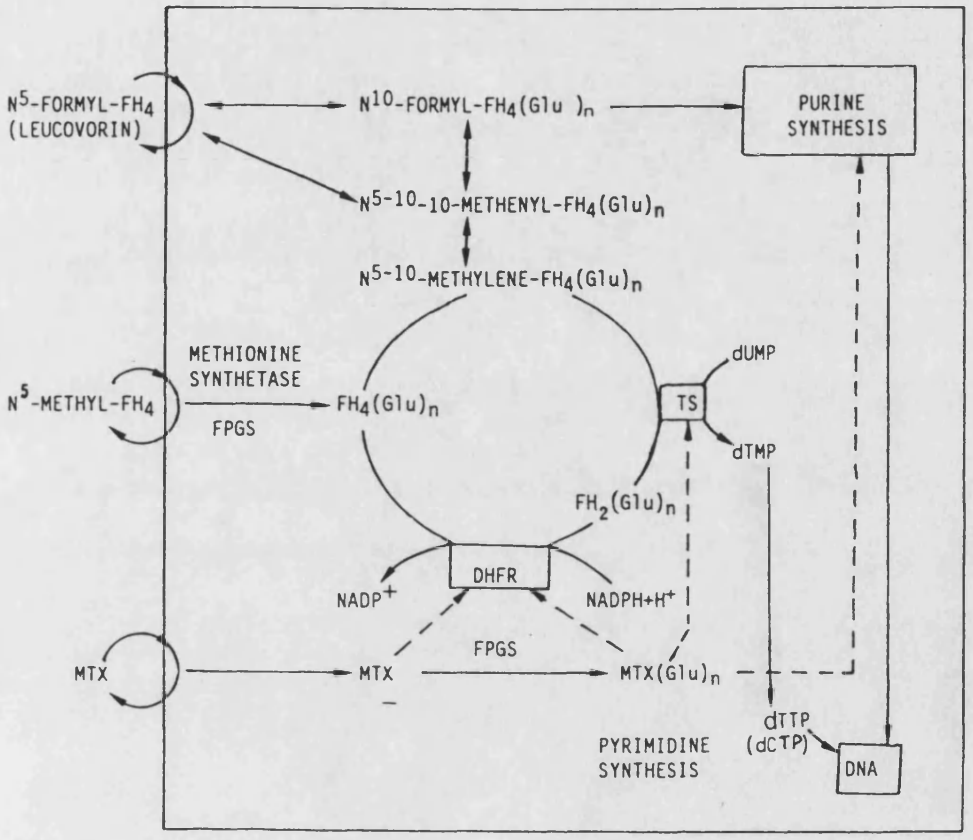


Methotrexate

MTX enters the cell via the carrier-mediated transport system for naturally occurring reduced folates (FH_4). In addition to this mechanism passive diffusion may be significant at elevated concentrations (above 20uM) (Warren et al, 1978). After entering the cell MTX binds with and competitively inhibits DHFR. This enzyme is responsible for maintaining intracellular folate (FH_4) levels by reducing dihydrofolates (FH_2) produced during thymidylate synthesis. The resulting high levels of FH_2 then compete with MTX to bind with DHFR (White, 1979). NADPH has been shown to form a ternary complex with MTX and DHFR exhibiting increased affinity (Blakely, 1984); inadequate levels of NADPH render MTX a reversible competitive inhibitor of DHFR (Kamen et al, 1983). The net result of the inhibition of DHFR is the depletion of FH_4 levels. Consequent reduced levels of N^{5-10}-methylene-FH_4 lead to a cessation of thymidylate synthesis; this occurs at MTX concentrations of $10^{-8}M$ (Chabner & Young, 1973). At MTX levels above $10^{-7}M$ depletion of N^{10}-formyl-FH_4 occurs resulting in the inhibition of purine synthesis (Zaharko et al, 1977). The lack of either thymidylate or purines leads to a cessation of DNA synthesis.

Intracellular folates are converted to polyglutamates primarily by folate polyglutamate synthetase with 5-7 extra glutamyl residues predominating (Covey, 1980). It has been shown by Mathews (1980) that the polyglutamated forms are actually the preferred substrates for folate requiring enzymes. Baugh et al (1973) showed that MTX was also a substrate for folate polyglutamate synthetase and later MTX-polygamma-glutamates were found in human liver (Jacobs et al, 1977). These derivatives appear to have at least equal affinity for DHFR (cf. MTX) (Jacobs et al, 1975) but dissociate at a slower rate suggesting

Figure 1.2 Mechanism of action of methotrexate (after Jolivet et al, 1983)



DHFR = dihydrofolate reductase

TS = thymidylate synthetase

FPGS = folate polyglutamate synthetase

FH_4 = Tetrahydrofolate

FH_2 = dihydrofolate

Glu = glutamate

dUMP = dioxyuridylate

dTMP = thymidylate

effectively reduced reversibility of inhibition. The polyglutamated forms of the drug also possess an increased intracellular half-life, and consequently prolonged inhibition of DHFR (Jolivet et al, 1982), which appears to be chain length dependent. In addition to DHFR, MTX-polyglutamates can also inhibit other folate-requiring enzymes eg. 5-aminoimidazole-4-carboxamide ribotide transformylase and glycinamide ribonucleotide transformylase both of which are involved in de novo purine synthesis and naturally utilise 10-methyltetrahydrofolate as a substrate. The polyglutamated forms of these folates appear to be preferred substrates for these enzymes (Allegra et al, 1985). Similarly MTX polyglutamates can competitively inhibit these enzymes as well as a thymidylate synthetase (Szeto et al, 1977). De novo purine synthesis can also be indirectly affected due to accumulation of oxidised folates caused by MTX inhibition of DHFR (Baggott et al, 1986).

1.4.3 Clinical Pharmacology

It is expected from clinical experience, cytotoxicity experiments using cultured cells and analysis of receptor-mediated folate/MTX transport (Kamen, 1987) that a plasma level exceeding 10^{-7}M would be cytotoxic (folate circulates at a concentration of approximately 10^{-8}M). At low doses (<50mg/m^2) MTX undergoes limited metabolism, however high dose therapy results in the formation of 7-OH-MTX (by hepatic aldehyde oxidase) which has a longer half-life than the parent compound; 23.8 hours as opposed to 2.1 hours (Breithaupt and Kuenzler, 1982). MTX is found to be protein-bound in plasma to a level of about 50% (Paxton, 1982) independent

of concentration (10^{-4}M to 10^{-9}M) inferring a high capacity, low affinity binding site. The metabolite, 7-OH-MTX, also binds to serum proteins (Lopez et al, 1986) to a level around 65%; Scatchard analysis implies two binding sites on human serum albumin, one being partially competitive with MTX.

The use of high dose MTX (HDMTX) (1-30g/m^2) followed by leucovorin (N^5-formyl-FH_4, folinic acid) rescue has become more frequent. The administration of leucovorin is followed by its rapid metabolism in the plasma to N^5-methyl-FH_4, the physiological circulating folate (Hamel et al, 1981) which can enter cells and replete the folate pools following metabolism by methionine synthetase. The use of HDMTX resulting in high plasma levels (10^{-4} to 10^{-5}M) for prolonged periods (12-36 hours) has a number of advantageous effects:

i) passive diffusion may overcome active transport defects.

ii) increased free intracellular MTX can overcome resistance due to unusually high levels of DHFR or altered enzyme binding.

iii) sustained, elevated plasma levels can promote increased polyglutamation.

iv) prolonged administration exposes more cells to MTX during DNA synthesis.

v) HDMTX may prevent or delay development of resistance which tends to occur in cells exposed to barely cytotoxic levels (Schrecker et al. 1971).

1.4.4 Cellular Resistance

MTX resistance may occur due to a variety of effects:

i) Decreased membrane transport.

Tumour cells in culture have been shown to become resistant to drug due to decreased affinity of the carrier for MTX in the active uptake system.

ii) Decreased affinity of DHFR for MTX.

Reduced binding affinity has been demonstrated in human cell lines with an associated reduction in cytotoxicity (Jackson & Niethammer, 1977)..

iii) Increased levels of DHFR.

Long term exposure of cells to differing levels of MTX has been shown to produce higher levels of DHFR possibly due to amplification of genes encoded for DHFR (Melera et al, 1980) which may be reversible (Schinke, 1986).

iv) Decreased polyglutamation

A cancer cell line in which decreased polyglutamation was in part causative of drug resistance has been described (Cowan & Jolivet, 1983) however it has not been extensively characterised.

v) Thymidylate synthetase activity.

A reduction in the rate of thymidylate synthesis has been shown to render cells less susceptible to MTX (Moran et al, 1979).

As previously outlined some of the mechanisms by which cellular resistance is thought to occur may be overcome by high dose MTX therapy followed by leucovorin rescue which has the additional benefit of exhibiting reduced toxicity.

The toxicity of MTX is associated with its anti-proliferative action exerted on growing tissues; mainly the bone marrow and the mucosae of the GI tract. In addition MTX is associated with hepatic (Lawrence et al, 1983) and CNS toxicity (Fritsch and Urban, 1984).

1.5 Human Serum Albumin

1.5.1 General Properties and Biological Function

Human serum albumin (HSA) is a plasma protein present at a level of about 42g per litre of plasma; this is equivalent to around 150g in the blood of a 70kg person. In addition to this circulating component about 230g exists in an exchangeable extravascular reservoir within the lymph spaces. The half-life of albumin is 19 days in humans; 14g being lost daily and replaced by hepatic synthesis.

The main functions of albumin are in the maintenance of fluid balance and the binding and transport of endogenous ligands. The former property arises from the relatively low molecular weight of HSA and its high concentration (60% of the total plasma protein). Albumin contributes about 80% of the colloidal osmotic pressure of plasma, which reduces the extravasation of fluid.

HSA is capable of binding a wide variety of ligands. This ability is derived in part from its molecular flexibility and also the fact that its chemical complexity allows both hydrophobic and ionic interactions with other molecules. The general properties of serum albumin have been reviewed by Peters (1985).

1.5.2 Structure

Primary Structure

Albumin is a secreted protein which characteristically has low contents of tryptophan and methionine and high levels of cysteine and charged amino acids (aspartate, glutamate, lysine and arginine) (Table 1.2). This results in a large number of ions per molecule at physiological pH and yields a net charge of -15 at pH7.

Table 1.2 Amino acid composition of Human Serum Albumin

aspartic acid	36	cysteine	35
asparagine	17	methionine	6
threonine	28	isoleucine	8
serine	24	leucine	61
glutamic acid	62	tyrosine	18
glutamine	20	phenylalanine	31
proline	24	lysine	59
glycine	12	histidine	16
alanine	62	tryptophan	1
valine	41	arginine	24

total number of residues	585
calculated net charge (pH 7)	-15
average residue weight	113.57
molecular weight	66241

Hunter & McDuffe (1959) discovered, by reduction and alkylation, that HSA was composed of a single chain. The sequence of the chain was later elucidated and published in 1975 (Brown, 1975, Behrens et al, 1975, Meloun et al, 1975).

Secondary Structure

Optical rotation measurements show HSA as a typical globular protein (at pH7) with an estimated 55% of the molecule as α-helix and 16% as β-pleated sheet.

Tertiary Structure

The presence of 17 disulphide bridges brings about the organisation of the molecule into a series of 9 loops each bound by two such bridges (see Figure 1.3). The nine loops are grouped in triplets (large-small-large) thus forming three domains; this may have arisen from an evolutionary precursor one third or one ninth the size of HSA.

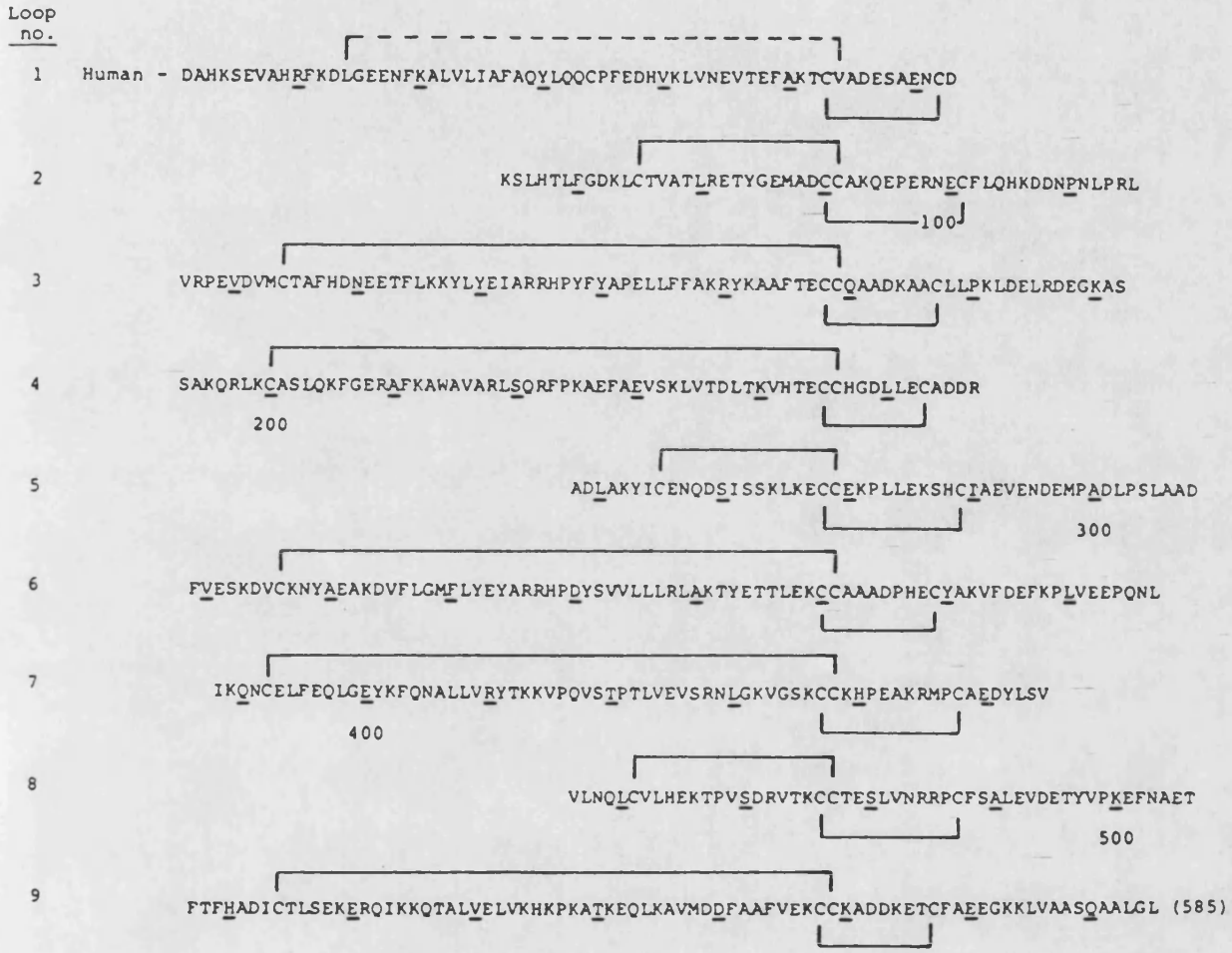
1.5.3 Physical Properties

HSA is an acidic, water-soluble, stable protein which has an isoelectric point (with bound fatty acid) at around pH4.8.

Size and Shape

Knowledge of the primary structure of HSA has enabled calculation of the precise molecular weight of the protein; ie. 66241. The higher values

Figure 1.3 Amino acid sequence and cysteine bridging of HSA



The one-letter code for amino acids is A,Ala; C,Cys; D,Asp; E,Glu; F,Phe; G,Gly; H,His; I,Ile; K,Lys; L,Leu; M,Met; N,Asn; P,Pro; Q,Gln; R,Arg; S,Ser; T,Thr; V,Val; W,Trp; Y,Tyr.

derived from physical measurements may arise due to the presence of adducts eg. fatty acids, carbohydrates, polymers and tightly bound water (c. 20 molecules) (Table 1.3).

The shape of HSA in solution is prolate ellipsoid with associated water molecules (both tightly and loosely bound) increasing its overall dimensions. At neutrality the molecule possesses maximal helical content (c. 55%). As pH is lowered (down to pH 1-2) it unfolds and elongates, complete unfolding being restricted by the disulphide bonds. At elevated pH there is only a slight drop in helical content; however, above pH 10, deamidation and disulphide interchange may occur. HSA also exhibits thermal stability and can withstand heating to 60°C for 10 hours (in the presence of stabilizers). The above effects tend to be reversible thus rendering HSA fairly resistant to denaturation. However prolonged or repeated heating or exposure to alkali will cause dimerisation, unfolding and eventual aggregation. Heating a solution at pH9 to 65°C causes polymerisation and loss of helix within one minute.

1.5.4 Metabolism

Biosynthesis

Albumin is synthesised in the liver and during this phase 19 ribosome units attach to one albumin mRNA forming a large polysome. A signal peptide is the first translated sequence and this is responsible for guiding the polysome to the endoplasmic reticulum (ER) and inserting the growing peptide chain through the ER membrane. The signal peptide is

Table 1.3 The physical properties of human serum albumin

Molecular weight	
i) from composition	66241
ii) from physical data	69000 (a)
Sedimentation constant, S_{20w}	
i) monomer	4.6×10^{8} (b)
ii) dimer	6.7×10^{13} (b)
Diffusion constant, D_{20w}	6.1×10^{7} (b)
Intrinsic viscosity	0.042 dl/g (a)
Overall dimensions	38 x 150 A (c)
Optical absorbance, E1%/1cm, 279nm	5.31 (d)

(a) Charlwood, 1961

(b) Oncley et al, 1947

(c) Hughes, 1954

(d) Edwards et al, 1969

cleaved off prior to completion of translation of the mRNA and detachment of the peptide chain from the ribosome, which is then followed by the linking of cysteine residues. This produces the peptide known as proalbumin (Judah et al, 1973) which differs from albumin in that it has a basic hexapeptide attached to the amino terminus. This peptide remains intact during the molecule's transit from the rough through the smooth endoplasmic reticulum to the Golgi apparatus. The hexapeptide is then cleaved prior to the release of albumin into the circulation; the entire secretory process taking 20-30 minutes.

The rate of albumin synthesis, maximal during periods of good health and nutrition, diminishes rapidly in malnutrition and disease states when the liver synthesises other proteins, eg. fibrinogen and orosomucoid.

The liver can increase albumin production by up to 100% during protein-losing enteropathy and nephrosis or following protein deprivation. The production of albumin can be stimulated by the anabolic hormones insulin and somatotropin (growth hormone).

Catabolism

The disappearance of albumin from the circulation is first order, a small quantity leaks into the GI tract from where its amino acids can be made available for reuse. Studies using bound radiolabelled oligosacharides (Yedgar et al, 1983, Baynes & Thorpe, 1981) show that albumin molecules are taken up by pinocytosis in cells throughout the body and degraded in the lysosomes. The rate of catabolism is directly related to the albumin plasma levels and may be increased by adrenal stress hormones. Mego

(1984) has suggested that modified albumin is degraded following pinocytosis, conversely unmodified molecules are released back into the circulation. It has also been suggested (Dammacco, 1980) that bound fatty acids may have some slowing effect on degradation.

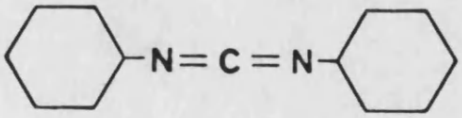
1.6 Carbodiimide reagents

1.6.1 Introduction

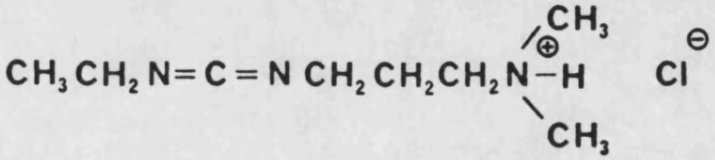
The carbodiimides are a group of bifunctional condensing reagents which were originally prepared about a century ago. A resurgence of interest in these compounds followed a review of their chemical and physical properties by Khorana (1953). The general structure of carbodiimides is as follows:

$$R-N=C=N-R'$$

where R and R' may be aromatic or aliphatic. Carbodiimide compounds may be soluble in organic solvents eg. dicyclohexylcarbodiimide (DCC)



or may be soluble in aqueous systems eg. 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (ECDI).



The versatility of carbodiimides as reagents for organic synthesis involves them in coupling reactions between such functional groups as carboxylic acids, phenols, amines, phosphates, alcohols and thiols resulting in the formation of amides, esters etc. They are particularly useful in the synthesis of peptides and nucleotides.

1.6.2 Synthesis

Carbodiimides themselves are synthesised from a variety of compounds including thioureas, ureas and isocyanates; the carbodiimides being formed by techniques such as dehydration or desulphurisation (Sheehan, 1961).

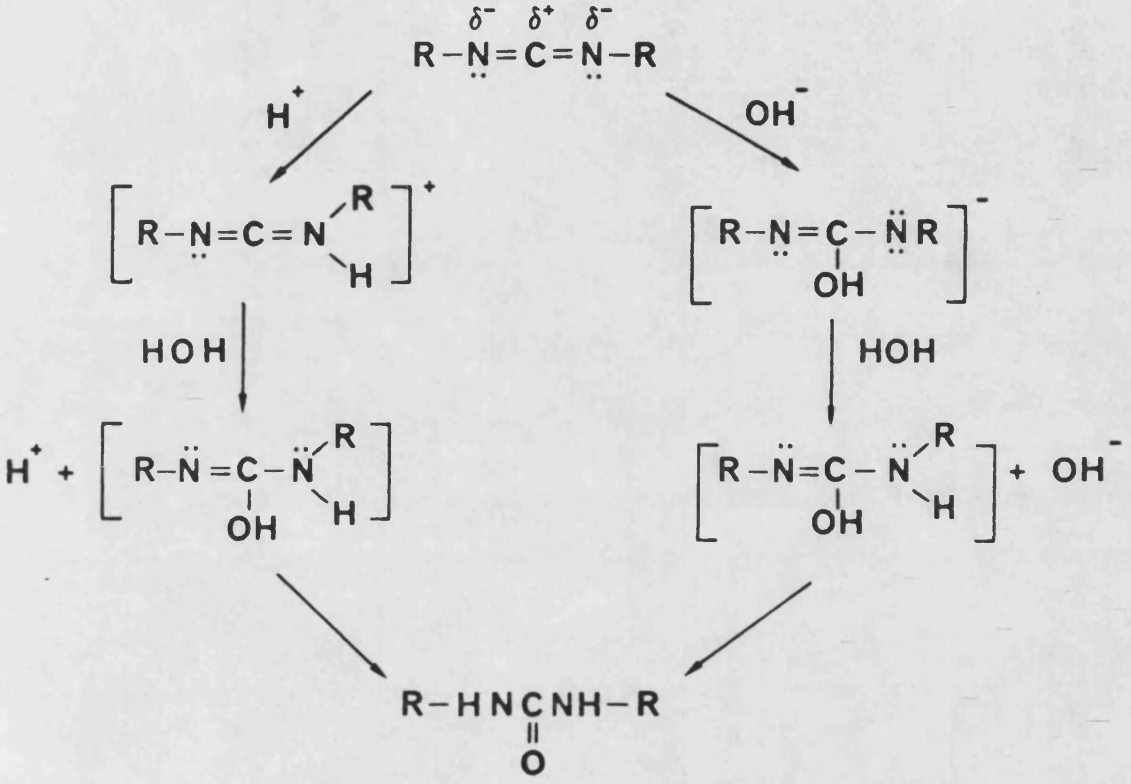
1.6.3 Reactions

Carbodiimides can, under the correct conditions, react with a variety of substances including alcohols, phenols, sulphonic acids, phosphate esters, inorganic acids, carboxylic acids and amino compounds (Kurzer and Douraghi-Zadeh, 1967). Carbodiimides undergo an addition reaction with water to form the corresponding urea; the reaction may be catalysed by both acid and alkali conditions (Scheme 1.1).

1.6.4 Peptide Bond Formation

One of the more useful attributes of the carbodiimides is their ability to unite free carboxyl and amino groups to form a peptide bond. This is a

Scheme 1.1 Reaction of carbodiimide reagents with water



one step synthesis which may be performed at room temperature with subsequent high yields (Sheehan and Hess, 1955). The latter authors reported that no racemisation occurred using an optically active dipeptide as the acylating agent.

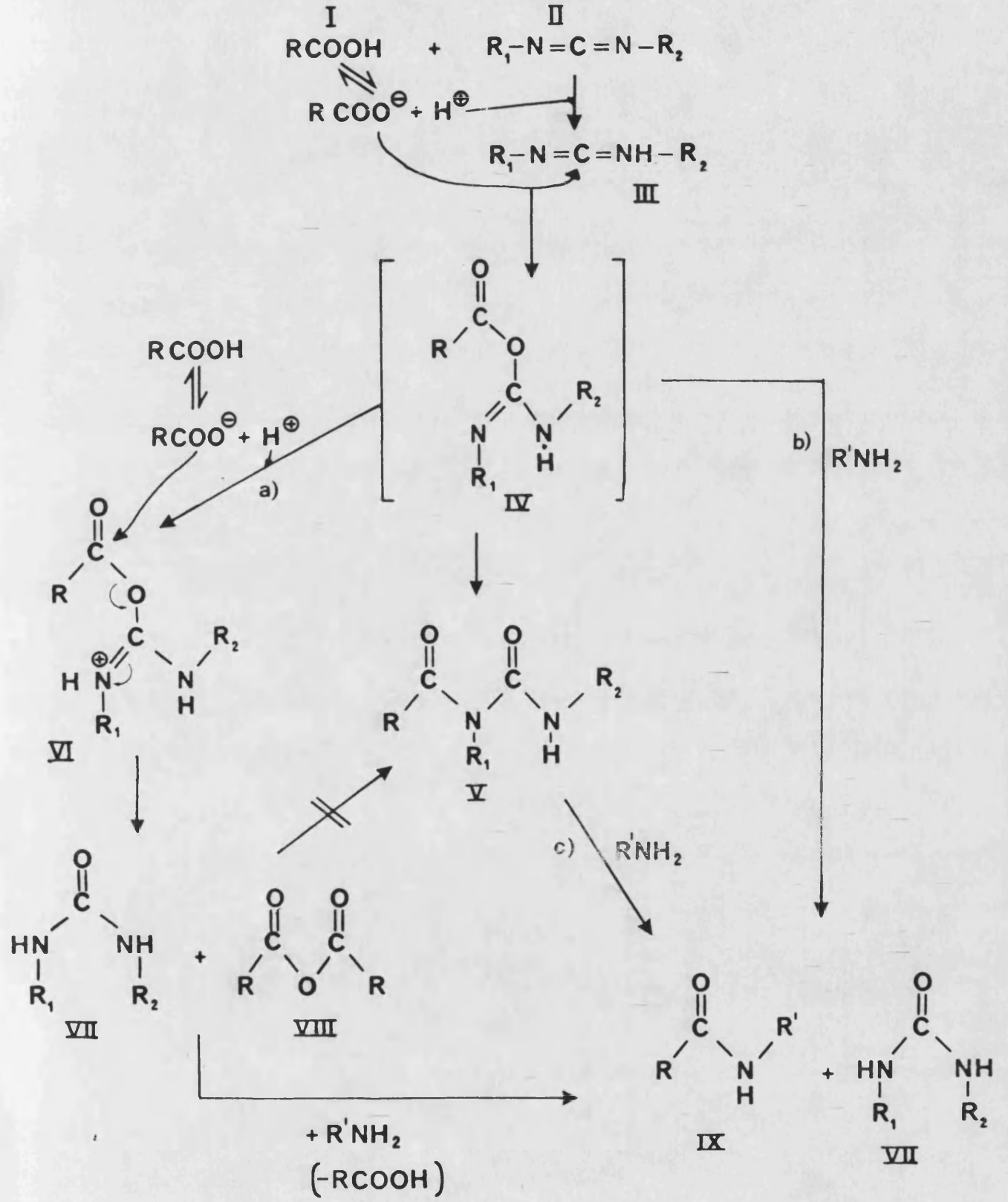
The general scheme of the reaction is as outlined in scheme 1.2. The formation of peptide bonds occurs via a 1,2 addition reaction due to the twinned double bonds of the carbodiimide. The first step is the addition of a proton to form compound (III) which is subsequently susceptible to attack by the acid anion forming the O-acylisourea (IV). The formation of a peptide bond could then proceed by one of three routes (Scheme 1.2):

a) a proton is attached to (IV) forming the cation (VI) which is then subject to attack by the acid anion forming the urea (VII) (corresponding to the carbodiimide (II)) and an anhydride (VIII). The latter is then open to nucleophillic attack by an amine; the symmetrical anhydride thus acts as the acylating agent resulting in the formation of a peptide bond, accompanied by the liberation of the free carboxylic acid.

b) The peptide link results from the direct nucleophillic attack upon the O-acylisourea (IV) by the primary amine with the concomitant formation of the appropriate urea (VII).

c) The O-acylisourea (IV) undergoes intramolecular rearrangement to form the N-acylurea (V) which could be attacked by an amine to produce the amide and urea derivatives.

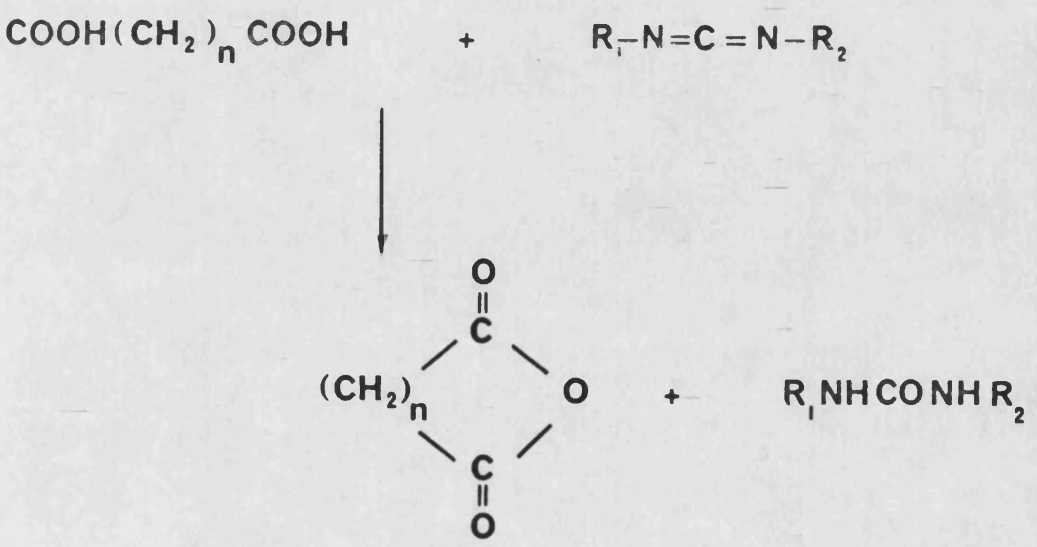
Scheme 1.2 Peptide bond formation using carbodiimide reagents



The formation of peptides via the stable N-acylurea (path c)) has been excluded by reaction kinetics experiments described by De Tar (1966).

Evidence in support of pathway b) was provided by Weetall and Weliky (1964) who linked amines to polymer bound carboxylic acids using DCC; thus preventing anhydride formation. Conversely Rebek and Feitter (1974) concluded that the anhydride is the acylating agent following solid phase peptide synthesis experiments with polymer-bound amines. They proposed that in solution the O-acylisourea is the acylating group, ie. path b), whereas it is the anhydride which acylates in the solid phase, ie. path a).

Dicarboxylic acids, eg. glutaric acid, can react with carbodiimides to form a cyclic anhydride and the disubstituted urea via pathway a) (Khorana, 1953). Cyclisation may occur following the first 1,2-addition (IV); the reaction being facilitated by the proximity of the second carboxylate group.



The anhydride thus formed would then be subject to nucleophillic attack (by an amine) as would intermediate VIII. The molecular rearrangement to the N-acylurea (V) predominates at elevated temperature; the production of this side product may be reduced by lowering the reaction temperature and by the presence of an amino group during the reaction (Bauminger and Wilcheck, 1980).

The use of insoluble carbodiimides eg. DCC, diethylcarbodiimide and diphenyl carbodiimide has become popular in non-aqueous peptide synthesis since the urea by-products tend to be insoluble and are readily removed by filtration. (Sheehan and Hess, 1955).

Water-soluble carbodiimides first developed by Sheehan and Hlakva (1956) also find applications in aqueous and non-aqueous peptide synthesis. This class of carbodiimides include tertiary and quaternary amine substituents being soluble in dilute acid and water respectively. These reagents give rise to water soluble ureas during peptide synthesis which can be removed from an organic environment by aqueous extraction; this can prove useful if the product and side product (urea) are both insoluble using water-insoluble CDI's (eg. as might occur during DCC coupling of high molecular weight peptides).

In addition to their uses in peptide synthesis, carbodiimide reagents have been used in the preparation of immunizing conjugates. The purpose of these systems is to enable antibodies to be elicited against small molecules (haptens) of molecular weight less than 1000. These haptens, which are not normally antigenic, are rendered so by covalently linking them to proteins or synthetic polypeptides (Erlanger, 1980). Serum

albumins have commonly been used as the carrier proteins in such systems, eg. bradykinin and angiotensin linked to rabbit serum albumin (Goodfriend, 1964), steroidal hormones (progesterone) linked to bovine serum albumin (Erlanger et al, 1959), α-melanocyte stimulating hormone linked to albumin (McGure, 1965) and prostaglandins bound to poly-L-lysine (Levine & Van Vunakis, 1970).

The use of water-soluble carbodiimides in such systems enables separation of the product from uncoupled hapten and urea by-products by dialysis or gel filtration (Bauminger & Wilchek, 1980). The amount of conjugated hapten can be estimated, following separation, by various techniques depending on the nature and pretreatment of the hapten, eg. spectrophotometry, radioactive tracer assay or quantitative analysis of the carrier, eg. amino acid analysis. As has been previously described carbodiimide reagents can react with a variety of functional groups (such as those present in albumins) and they have been shown to react with protein-tyrosine residues (Carraway & Koshland, 1968) and sulphydryl groups (Carraway and Triplett, 1970) which may affect the antigenic potential of the product.

Carbodiimides have also found application in the determination of carboxyl groups in proteins by conjugating them with radiolabelled ^{14}C-glycinamide (Carraway & Koshland, 1972). This procedure also yielded information on the accessibility of carboxyl groups by reaction in the presence and absence of high concentrations of urea or guanidine (denaturing reagents).

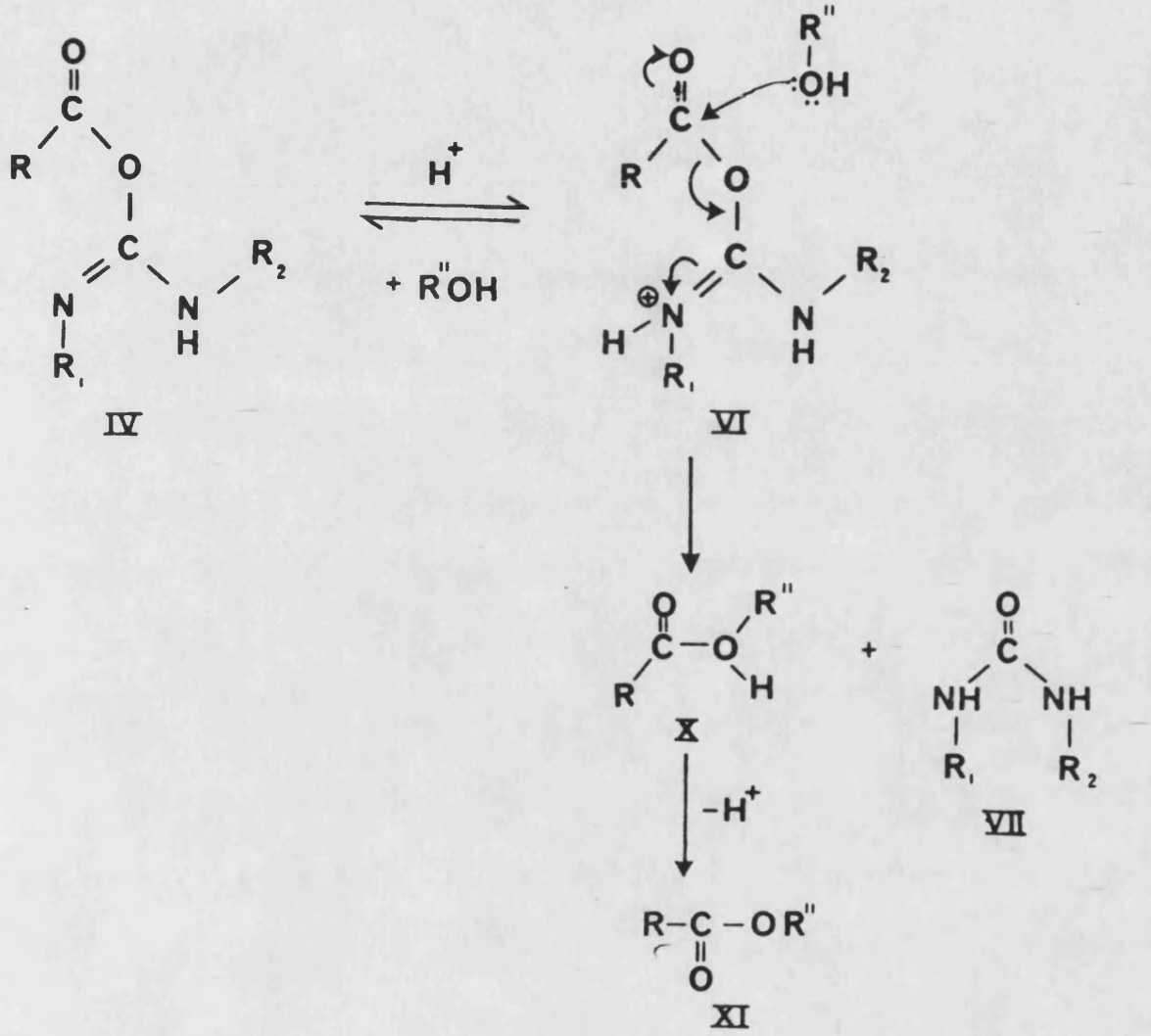
The desired reaction in conjugation procedures involving hapten, carbodiimide and protein results in the formation of an amide bond, ie. a

hapten molecule containing a carboxylic acid residue may combine with one of the lysine residues of the protein (which have pendant amine groups). In addition to such amide bonds, ester linkages may also be formed between hapten carboxyl groups and hydroxyl groups present in serine and threonine residues of the protein. Esterification may occur via the O-acylisourea (IV) or the anhydride (VIII) (March) (Scheme 1.3). ii) Alcoholysis of anhydrides

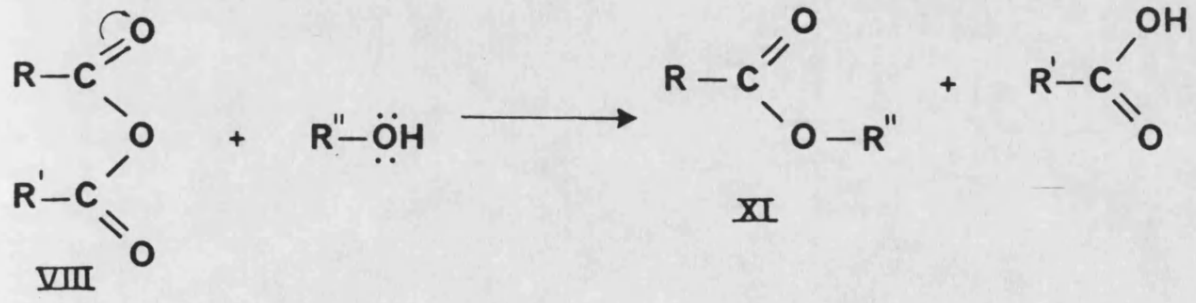
The panoply of amino acid residues present in proteins, with their associated free functional groups, render inter & intramolecular cross-linking a distinct possibility in carbodiimide-mediated reactions. Timkovich (1977) has reported polymerisation in four different proteins; the extent of the polymerisation was significantly reduced by the presence of a nucleophile at high concentration. The amino acid residues which are proposed to be involved in cross-linking reactions (with O-acylisoureas) are tyrosine, serine or threonine (hydroxyl residues forming esters), glutamic acid (carboxylic acid residues forming anhydrides) and lysine (amine residues forming amide bonds). It was concluded that the polymers formed in this experiment (as detected by gel electrophoresis) could be readily separated by gel filtration and using this technique a level of 20% polymerisation was determined in the absence of nucleophile. The addition of nucleophile significantly reduced the level of polymerisation in the case of myoglobin & cytochrome C (down to 6%) and prevented polymerisation in the lysozyme & ribonuclease reactions.

Scheme 1.3 Formation of esters

i) esterification via O-acylisourea



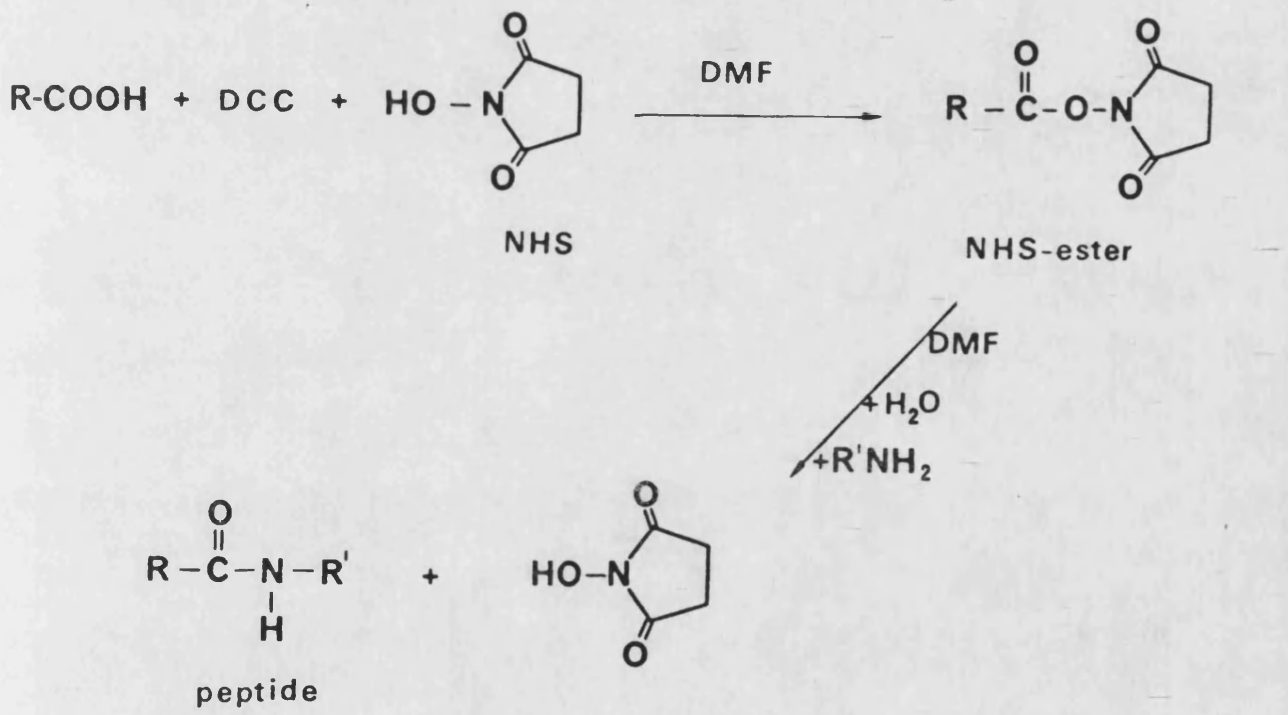
ii) alcoholysis of the anhydride



1.6.5 Peptide bond formation by activated esters

The formation of peptide bonds has also been achieved using carbodiimide reagents via an activated intermediate. Anderson et al (1964) used esters of N-hydroxysuccinimide (NHS) in peptide synthesis; the esters are the product of a carbodiimide-mediated reaction between a carboxylic acid and NHS (NHS may be produced by the fusion of succinic anhydride and hydroxylamine HCl) (Scheme 1.4).

Scheme 1.4 Peptide bond formation using N-hydroxysuccinimide



The reaction is performed in a non-aqueous environment, eg. DMF (Kulkarni et al 1981), and proceeds via the O-acylisourea with the insoluble urea derivative of DCC ultimately being precipitated permitting its removal by filtration. The peptide link with a free amine could subsequently be effected by reaction with the activated ester in a semi-aqueous environment.

CHAPTER TWO: SYNTHESIS AND CHARACTERISATION OF MTX-HSA CONJUGATES

2.1 Introduction

2.1.1 Gel Permeation Chromatography

2.1.1.1 Introduction

Gel permeation chromatography (GPC) is a term which was proposed by Moore in 1964 to describe the separation and characterisation of protein components using porous gel matrices. A number of workers have used a variety of terms to describe the same phenomenon i.e. gel filtration (Porath and Flodin, 1959), molecular sieve filtration (Fasold, 1975), restricted diffusion chromatography (Steere and Ackers, 1962), molecular sieve chromatography (Hjerten and Mosbach, 1962) exclusion chromatography (Pedersen, 1962) and simply gel chromatography (Determann, 1964).

The main component of a GPC system is a swollen gel matrix through which a liquid mobile phase is pumped. The system is capable of separation according to molecular size. Molecules with a larger hydrodynamic volume being eluted before those occupying a smaller volume. The primary process involved is the diffusional partitioning of solute molecules between a mobile solvent phase and the interior solvent spaces within the porous particles comprising the stationary phase; large molecules are sterically excluded from the matrix pores and permeate more rapidly through the column with the mobile phase.

A GPC system comprises the following:

i) a column containing the swollen gel matrix

ii) mobile phase pumped at a constant rate through the column

iii) flow detector and/or fraction collector

The gel is composed of a 3-dimensional cross-linked matrix which is in a swollen state in the mobile phase. Ideally the gel should possess the following properties:-

i) Inert character

chemical interaction between solute and matrix may lead to irreversible binding or to modification of labile substances.

ii) Chemical stability

gels need to be stable over a range of pH and temperature without leaching material and should be capable of being used repeatedly over an extended time period.

iii) Low content of ionic groups

to avoid ion exchange effects

iv) Narrow particle size distribution

particle size should be carefully controlled; small particles with a narrow size distribution give better resolution.

v) Mechanical rigidity

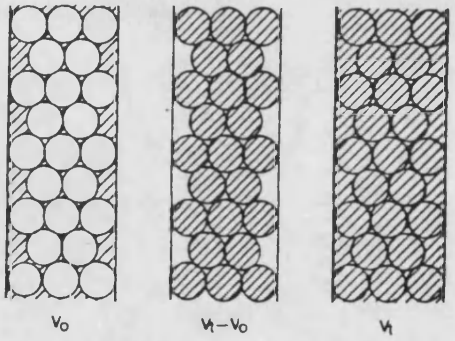
softer gels may be deformed and thus limit flow rates.

The volume of the chromatographic bed, Vt, consists of the following components.

$$Vt = Vo + Vi + Vg \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where Vt = total volume of the column, Vo = void volume, Vi = internal volume and Vg = gel matrix volume. Vi + Vg = Vt - Vo (see Figure 2.1)

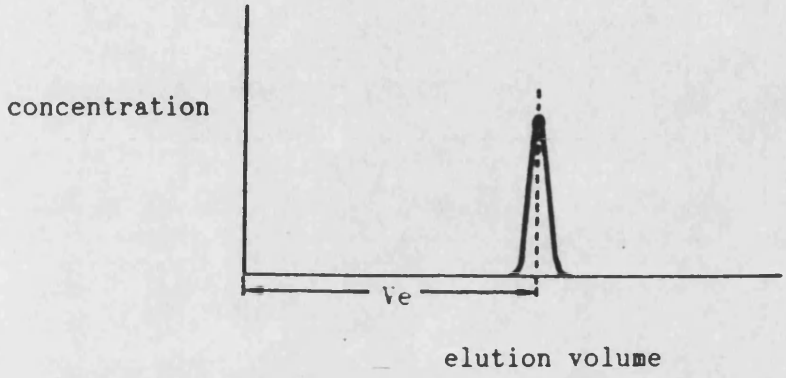
Figure 2.1 Diagrammatic representation of Vt and Vo



The void volume (Vo) represents the elution volume of a solute which is totally excluded from the gel matrix (ie. elutes with the solvent front) and is equivalent to the volume of liquid in the interstitial spaces between the particles.

In a chromatographic experiment a sample is applied to the gel column and the concentration of solute in the effluent is monitored. When this is plotted against the volume of eluant collected it yields the elution curve (Figure 2.2).

Figure 2.2 Elution curve from a GPC column



The elution volume (Ve) can be used to characterise the behaviour of a given solute in a given system under controlled conditions and should ideally be independent of flow rate.

The solute in a gel/solvent system is distributed by diffusion between solvent regions inside and outside the gel. At equilibrium a partition isotherm can be deduced defining the relationship between the weight of solute (Qi) inside the gel and the solute concentration (C) in the void space.

$$Qi = f(C) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

The distribution coefficient (partition coefficient) referred to internal solvent volume, Kd, is a ratio of the mass of solvent in the gel phase per unit volume and the concentration in the external phase.

$$Qi/Vi = Kd.C \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

Or, $$Kd = Qi/Vi.C \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

Kd is thus a dimensionless measure of the extent of solute penetration within the available interior solvent region of the gel.

$$Kd = Vp/Vi \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (5)$$

where Vp = penetrable volume occupied by solute molecules at equilibrium.

Therefore, $$Kd = (Ve - Vo)/Vi \quad \ldots\ldots\ldots\ldots\ldots \quad (6)$$

An alternative distribution coefficient, Kav, can be calculated with reference to the total volume of the gel. In this case Vx is considered as the stationary phase where

$$Vx = Vt - Vo = Vi + Vg \quad \ldots\ldots\ldots\ldots \quad (7)$$

Then, $$Qi = Kav.Vx.C$$

therefore, $$Kd.Vi = Kav.Vx$$

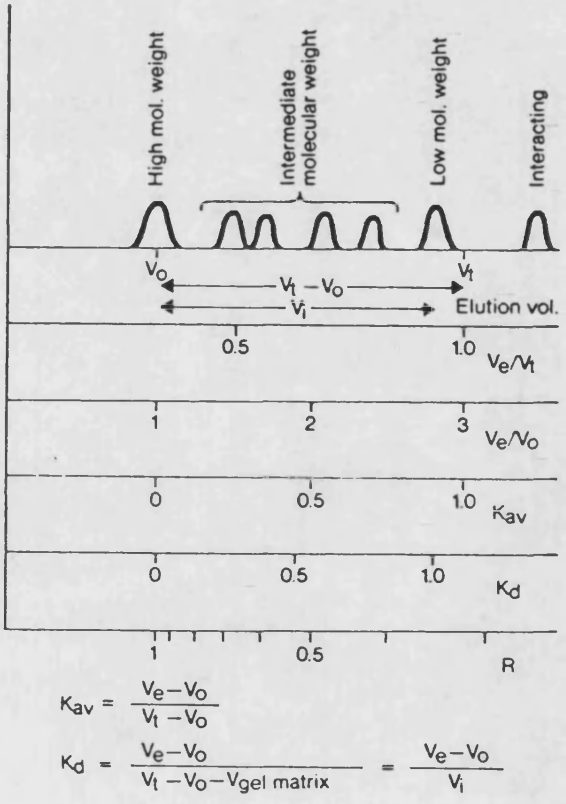
or, $$Kd/Kav = Vx/Vi$$

which is a constant independent of solute concentration.

Therefore, Kav = (Ve - Vo)/(Vt - Vo) ..... (8)

Kav can thus be used to characterise the elution of solutes in GPC and indicates the fraction of the stationary phase that is available to the solute. It is not a true distribution coefficient since it incorporates the volume of impenetrable gel matrix, it is however more convenient to derive since determination of Vi is difficult. Kav can be calculated from knowledge of the total column volume, the void volume and the elution volume (Figure 2.3).

Figure 2.3 Relationship between expressions used for normalising elution behaviour



2.1.1.2 Solute - matrix interaction

The matrix material is selected to be inert, however some substances may still interact. In aqueous systems the main types of interaction are ion-ion interactions and Van der Waals interactions. Van der Waals interactions lead to common phenomena known as hydrogen bonding and hydrophobic bonding. In organic solvents hydrogen bonding and hydrophilic interactions may occur.

Two types of ionic interactions are encountered

i) Direct interaction with trace amounts of charged groups present on the matrix - mainly carboxyl but also sulphate groups on agarose columns.

ii) Donnan effect. A potential builds up due to partitioning of low molecular weight counter-ions into the gel from an eluant containing a charged macromolecule.

The effects of both types of ionic binding decrease with increasing ionic strength. In practice ionic strengths greater than 0.02M minimise the effects of ionic binding.

Hydrophobic interactions were initially noted in relation to aromatic substances by Gelotte in 1960. However aliphatic compounds have also

been shown to interact with the matrix (Marsden, 1965). All gel matrices have potential sites for hydrophobic interaction and these sites have been identified in some cases, eg. the cross-linking bridges in Sephadex (Determann and Walter, 1968). The extent of the hydrophobic interaction tends to increase with increasing ionic strength.

Hydrophobic interaction can only occur between accessible sites of the solute and the gel matrix. In the case of proteins hydrophobic regions tend to be within the interior of their tertiary structure and consequently cannot bind with hydrophobic gel sites. The primary hydrophobic binding site of Sephadex and Sepharose gels are the cross-linking bridges which are surrounded by matrix chains and are consequently inaccessible to compact, sterically excluded molecules, eg. proteins. The interaction and adsorption of intact proteins is thus rarely observed. However the probability of such phenomena occurring with low molecular weight compounds is much greater. Molecules which interact are often eluted as sharp, well defined peaks; thus the effect can be used to separate molecules which may be difficult to resolve. The interaction of purines with Sephadex has been employed in the separation of folic acid and derivatives (including polyglutamates) using Sephadex G10 (Kas and Cerna, 1976).

2.1.1.3 Measurement of column efficiency

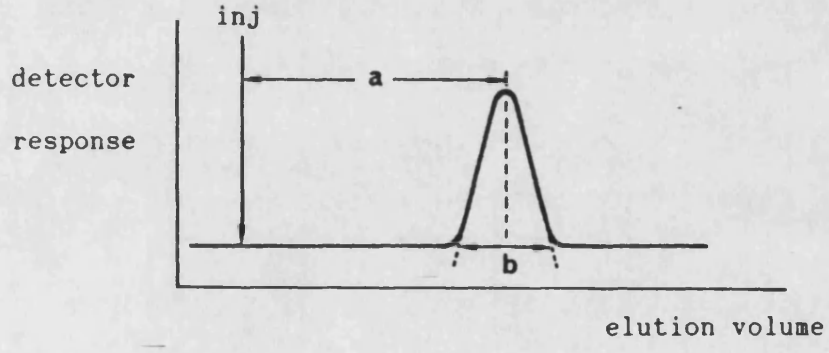
An indication of the performance of a column can be obtained by determining the number of theoretical plates, which is a measure of the amount of zone broadening caused by the column. The height equivalent to a theoretical plate (HETP) can be determined by calculating the number of

theoretical plates (N) (Figure 2.4).

HETP = L/N where L = length of column in cm.

HETP is a measure of column efficiency allowing comparison between columns of differing length.

Figure 2.4 Calculation of the number of theoretical plates from chromatogram



$$N = 16(a/b)^2$$

where a = distance from injection to peak maximum and b = length of baseline limited by tangents to the peak.

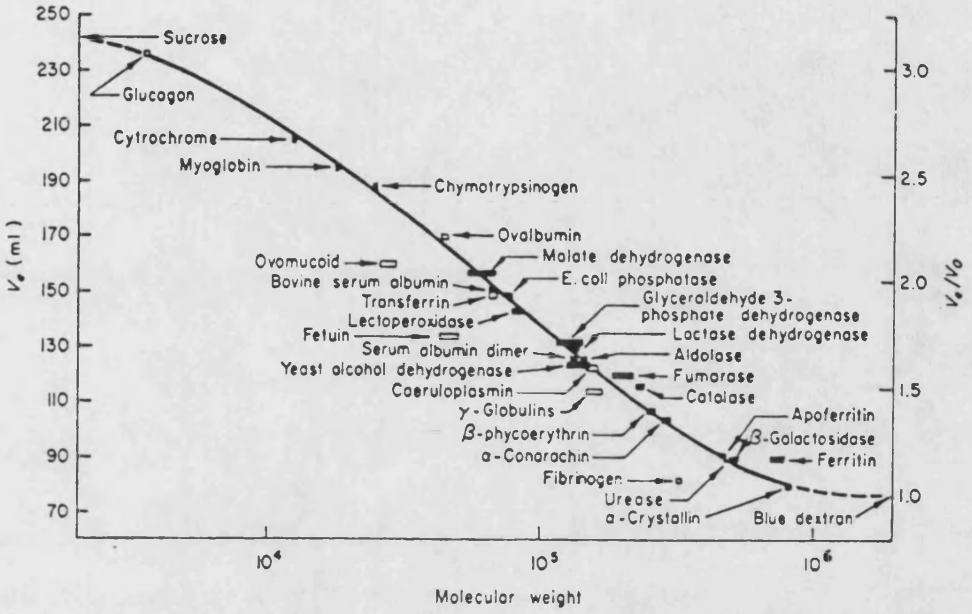
Plate number is a measure of the amount of zone broadening caused by a column and is dependant upon column length.

2.1.1.4 Determination of molecular weight

One of the most important applications of GPC is molecular weight determination, particularly of proteins. The results obtained are reliable, accurate and compare favourably with other techniques eg. viscosity, diffusion and light scattering. A number of workers (eg Whitaker, 1963, Determann and Michel, 1966) have indicated that for most proteins there is a close correlation between molecular weight (molecular radius) and elution behaviour as determined by Kav values. The partition coefficient (Kav) corresponds to the distribution of solute within the gel matrix at thermodynamic equilibrium, this is representative of the steric constraints leading to exclusion which are determined by molecular size and shape. A GPC system can be used for molecular weight determinations following calibration of the columns using suitable materials of defined physical properties. Molecular weight determinations have also been performed in the presence of denaturing agents such as urea, guanidine hydrochloride or sodium dodecyl sulphate (Fish et al, 1969, Hung et al, 1977) thus minimizing the effects of tertiary or quaternary protein structure on elution.

Andrews (1965) showed using Sephadex G200, that a relationship existed between the partition coefficient and the molecular weight of a number of proteins (Figure 2.5).

Figure 2.5 Logarithmic calibration plot for proteins on Sephadex G200



The following relationship was discovered empirically using semi-logarithmic plots:

$$Kav = -A. \log M + B$$

where A and B are constants and M is the molecular weight.

This equation/calibration is particularly applicable for the molecular weight determination of proteins having a similar structure to those used for the column calibration.

Calculation of Molecular Weight Averages

Number and weight averages can be calculated from a normalised GPC chromatogram using definitions which were developed for polymer chains.

If i = degree of polymerisation

Na = Avogadro's number

Mi = molecular weight of polymer chain having a degree of polymerisation i

Ni = number of molecules with degree of polymerisation i

ni = number fraction = $Ni/\sum Ni$ ..... (9)

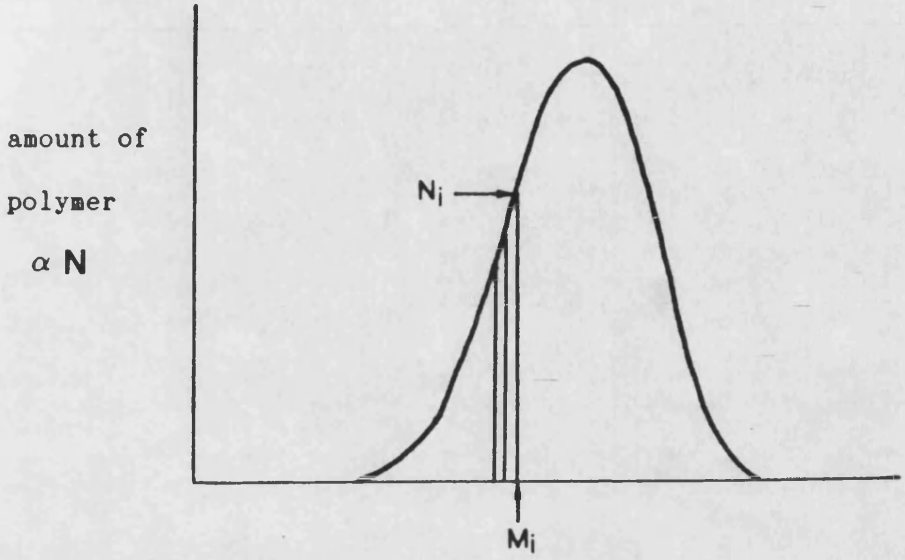
Wi = weight of molecules with degree of polymerisation i

wi = weight fraction = $Wi/\sum Wi$ ..... (10)

Then $Wi = Ni.Mi/Na$ ..... (11)

A chromatogram is essentially a plot of detector response (which is proportional to the number of molecules) against the retention volume (proportional to the molecular weight) (Figure 2.6).

Figure 2.6 Normalisation of a chromatogram



The chromatogram can be converted into a series of vertical lines and digitised. The amount of polymer can be expressed by number or by weight; if the data are converted to number or weight fractions then the chromatogram is said to have been normalised and is independent of the absolute amount. Normalisation can be achieved by dividing the digitised heights by the total area under the curve.

Number average molecular weight, ($\bar{M}n$)

$\bar{M}n$ = the sum of the products of the molecular weight of each fraction multiplied by its mole fraction

ie. $$\bar{M}n = \Sigma\, ni.Mi$$

then $$\bar{M}n = \Sigma\, Ni.Mi / \Sigma\, Ni$$

Weight average molecular weight, ($\bar{M}w$)

$\bar{M}w$ = sum of the products of the molecular weight of each fraction multiplied by its weight fraction

ie. $$\bar{M}w = \lesssim wi.Mi$$

since $$wi = Ni.Mi / \lesssim Ni.Mi$$

then $$\bar{M}w = \lesssim Ni.Mi^2 / \lesssim Ni.Mi$$

The relationship between Mn and Mw can be defined from the variance of the number distribution:

$$\sigma^2 = \lesssim Ni(Mi - \bar{M}n)^2 / \lesssim Ni$$

This may be rearranged to

$$\sigma^2 = (\bar{M}w.\bar{M}n) - \bar{M}n^2 = \bar{M}n^2(\bar{M}w/\bar{M}n - 1)$$

therefore $$\bar{M}w/\bar{M}n = 1 + \sigma^2/\bar{M}n^2$$

Since σ can only be zero or positive then the ratio $\bar{M}w/\bar{M}n$ must be greater or equal to unity. When σ is zero then the polymer is monodisperse. The ratio of $\bar{M}w/\bar{M}n$ is thus a measure of the spread of the molecular range within the polymer and is termed the polydispersity index. Mw by definition is always greater than $\bar{M}n$. The lower value produced by the number distribution arises from the fact that a given weight fraction of small molecules contains a large number of molecules whereas the same weight fraction of large molecules contains a smaller number of molecules.

2.2 Synthesis of MTX-HSA Conjugates

2.2.1 Introduction

The reaction used to conjugate MTX to HSA in this series of experiments was similar to that used by Kulkarni et al (1981) and Chu and Whiteley (1977). The reaction was performed in phosphate-buffered saline (PBS) pH 7.4 (I = 0.29 or 0.15M, 0.05M total phosphate). This pH was chosen as a compromise since the ECDI link to the acid residue is promoted by hydrogen ions and the lysine residues (on HSA) are more reactive at elevated pH where unprotonated amine groups occur (pKa = 9.8). The reaction was terminated by fractionation on a GPC column. The molar conjugation ratio (MCR) of MTX to HSA was determined by spectrophotometry and subsequently the stability of MTX-HSA conjugates was assayed by FPLC.

The reaction mixture was prepared by taking aliquots of concentrated reactant solutions (in PBS) and making up to the desired volume with PBS. Solutions of HSA (20mg/ml) and MTX (20mg/ml free acid) were either freshly produced or recently produced and stored at -20°C. The ECDI solution was prepared immediately before use. The reaction was allowed to proceed for up to 24 hours prior to termination.

The separation of the high molecular weight conjugate from the low molecular weight reactants (MTX, ECDI) and by-products (urea derivatives) was achieved using GPC. The gels employed were Sephadex G15, G25 & G50 (exclusion limits 1,500, 5,000 & 30,000 Daltons respectively (Pharmacia)) packed into 1.6cm x 75cm or 2.6cm x 35cm columns. The eluent (PBS pH7.4) was pumped using a peristaltic pump at a flow rate dependant on the column

and the eluate monitored by flow UV spectrophotometry at 254 or 280mm. The reaction mixture was applied to the column via a calibrated loop (1 or 5ml) and the conjugate, which eluted as a yellow band with the void volume, was collected into either a 25 or 100ml volumetric flask.

The molar conjugation ratio (MCR) was determined by UV assay at 376nm to estimate the amount of MTX present; from this and the known amount of HSA applied to the column the MCR was calculated, assuming 100% recovery from the column (see section 2.2.3).

2.2.2 Loop Calibration

The application of sample to a gel column may be achieved either directly onto the column using a syringe or via a loop system as was used in this study. The advantages of the latter method are:

i) the sample can be applied without significantly interrupting the flow of mobile phase.

ii) a knownvolume of sample can be applied using a calibrated loop.

The injection system was based around two four-way valves with two loops operating on the same apparatus. The approximate volumes of the loops (ie 1 & 5 ml) were determined from the internal dimension of the tubing and its length; the loops were then calibrated in situ in the following manner:

i) a solution of MTX (0.5mg/ml in PBS) was injected into the appropriate loop in the load position.

ii) the valves were turned to the inject position and the loops flushed out with mobile phase and collected directly into an appropriate volumetric flask; the loop having been flushed with 15-20 loop volumes of PBS.

iii) standards were produced by pipetting the appropriate volume of MTX solution into a volumetric flask.

iv) the solutions were assayed by UV spectrophotometry at 370nm, sufficient replicates being determined until successive readings were ± 0.002 absorbance units (typically 3-4 readings)

Table 2.1 Calibration of injection loops used with Sephadex columns

Nominal loop volume	Test sample reading ± sd (n)	Standard reading ± sd (n)	Actual loop volume
1ml (diluted to 25ml)	0.3475 ± 0.0054(4)	0.3334 ± 0.0042(2)	1.04 ml
5ml (diluted to 100ml)	0.4128 ± 0.0022(6)	0.4014 ± 0.0031(3)	5.13 ml

2.2.3 The yield of HSA from Sephadex columns

The determination of the yield of HSA from Sephadex columns was important since the MCR calculation was based on the assumption that all of the applied conjugate was separated from the free MTX and was subsequently collected for assay. The experiment was conducted using two columns in series, the first containing Sephadex G15 (70cm x 1.6cm) and the second Sephadex G50 (20cm x 1.6cm). The gels had been used previously but had been flushed with at least three column volumes of 0.2M NaOH and then equilibrated with distilled water. Water was used as the mobile phase in order to encourage binding (ionic interaction being reduced with increasing ionic strength). Flow spectrophotometers were placed in line between the two columns and after the second column thus enabling monitoring of the eluant from both columns.

The elution characteristics of HSA solution (1mg/ml) were compared to that of a blue dextran solution with the result that both compounds eluted from both columns with identical retention volumes. Since blue dextran has a molecular weight of 2,000,000 and is used to determine the void volume in GPC it confirmed that HSA eluted with the void volume from both grades of Sephadex.

In the adsorption experiments 1ml samples of HSA solutions (5 & 10mg/ml) were applied to the column (flow rate 2.55ml/min, H_2O) and samples of the effluent were collected. 6 x 10ml samples prior to the HSA peak were collected and then the peak (baseline to baseline) was collected in a 25ml volumetric flask (test sample) followed by further 10ml samples. The samples were the analysed at 279nm against water. Standard samples were

prepared by pipetting 1ml of sample solution and making up to 25ml in volumetric flasks (standard sample) and also by flushing the contents of the loaded 1ml loop directly into a 25ml volumetric flask (loop sample).

Recovery of HSA (4.9mg/ml) from a Sephadex G-15 column (duplicate samples)

mean test sample reading = 0.110 (±0.002)

mean loop sample reading = 0.111 (±0.001) (1.04ml)

mean standard reading (1ml) = 0.108 (±0.001) corr. to 1.04ml = 0.112

yield of HSA as % of loop sample = 99.1%

The absorbances of the other samples were found not to differ significantly from the blank.

The experiment was repeated using HSA at a concentration of 9.9mg/ml with the following results:-

mean test sample reading = 0.218 (±0.001)

mean loop sample reading = 0.224 (±0.001) (1.04ml)

mean standard reading (1ml) = 0.213 (±0.001) corr. to 1.04ml = 0.222

yield of HSA as % of loop sample = 97.3%.

The results indicated that the yield of HSA from the column was virtually 100% of that applied. However the accuracy of the experiment was limited by the sensitivity of the assay. It was thought reasonable to assume a yield of 100% for calculation of the molar conjugation ratios.

2.2.4 Experiment to determine the separation efficiency of a Sephadex column

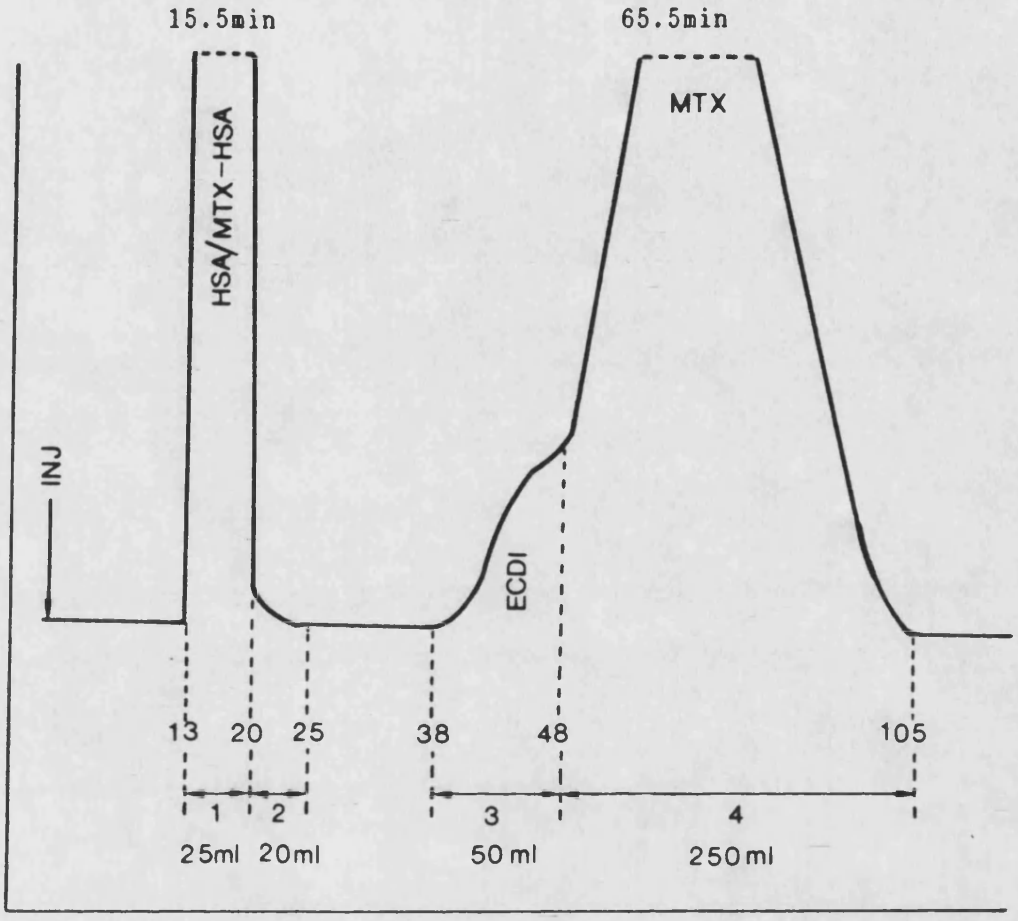
Since the conjugation of MTX to HSA requires an excess of drug it was important to ensure that the GPC column employed to separate the high molecular weight conjugate from the low molecular weight drug and by-products did so efficiently. The following reaction mixtures were consequently set up (Table 2.2):

Table 2.2

Reactant	Concentration	Volume (ml)			
		A	B	C	D
HSA	20mg/ml	1.5	1.5	1.5	-
MTX	20mg/ml	0.6	0.6	-	0.6
ECDI	25mg/ml	0.6	-	0.6	0.6
PBS	-	0.3	0.9	0.9	1.8
Total volume		3.0	3.0	3.0	3.0
Molar ratio HSA:MTX:ECDI		1:58.3:174.9	1:58.3:0	1:0:174.9	0:58.3:174.9

The samples were incubated at ambient temperature overnight and then 1.04ml aliquots were fractionated on a Sephadex G50 column (2.6cm x 38cm) the output being monitored at 254nm. The following fractions were collected as indicated on the chromatogram obtained for sample A (Figure 2.7).

Figure 2.7 Separation of reaction mixture on a Sephadex G50 column



The fractions were all assayed using a scanning spectrophotometer and absorbance readings taken at specific wavelengths ie. 230, 262, 279, 306 and 376nm thus enabling identification of each fraction from the differences in UV spectra (Table 2.3).

Table 2.3 Retention times (min) and UV absorption maxima (nm) of fractions taken from Sephadex fractionation experiment

Sample	Fraction number			
	1	2	3	4
A	15.5 min 236,262,306,376	- 223,262,303,373	- 226	65.5 min 226,258,303,373
B	15.9 min 231,279	- 223,279	- -	66.0 min 223,259,303,370
C	15.5 min 231,279	- 223,279	46.5 min 252	- -
D	- -	- -	47.5 min 233	64.5 min 226,259,303,370

Fraction 1 corresponded to material eluting with the void volume (ie with blue dextran) and represented substances with a molecular weight above around 50,000, (Kav = 0) ie. excluded material.

The retention time of fraction 3 corresponded to that of ECDI (or its urea derivative) and had a Kav of 0.8. The final fraction (4) whose

chromatographic characteristics corresponded to MTX had a Kav value of 1.2 which indicated some column interaction which was advantageous in a separation of this nature.

The UV analysis from sample A (ie. HSA/ECDI/MTX) fraction 1 showed that it had a fairly high content of MTX; this was not pure MTX since there was a slight shoulder on the absorption spectrum close to 280nm and also a spectral shift of the λ max values. The absorbance ratio (306nm/376nm) value of 3.5, as opposed to 3.1 for MTX, further suggested that this fraction was a high molecular weight derivative of MTX, ie. MTX-HSA. The MCR of this conjugate was calculated as 14.2 moles MTX per mole HSA. Fraction 2 from sample A yielded a similar spectrum but at a much lower concentration and showed that slightly over 98% of the MTX-HSA conjugate was collected in the first 25ml sample (ie. fraction 1). Fraction 3 yielded a spectrum with a λmax at 230nm with no detectable MTX present; this was presumed to be the urea derivative of ECDI. The final fraction (4) had a UV spectrum which was concordant with that of MTX ie. 303/373 ration of 3.1 with no bathochromic shift indicating that it was unmodified MTX. The total yield of MTX from the column was 96% indicating that very little had been adsorbed (the slight deviation from 100% could be accounted for by the method of determination).

Sample B (ie. HSA/MTX) separated as two discrete peaks, fractions 1 & 2 corresponding to HSA alone (no MTX detectable) and fraction 4 as MTX (symmetrical peak) with 303/373 ratio = 3.1. The yield of HSA was 105%, 99% of which was collected in fraction 1. The yield of MTX was 102% all of which was in fraction 4 which was collected in a volume of 250ml. The fractionation of sample C (ie HSA/ECDI) resulted in two peaks, the

first being identified as HSA (fraction 1 & 2) (110% yield) and the second (fraction 3) eluting at the appropriate time (ie 47 mins) and giving the appropriate spectrum of ECDI in buffer. The eluant collected at times corresponding to the retention time of MTX showed only a baseline when analysed by UV was not significantly different from the blank indicating that no material eluted in this fraction.

The chromatogram produced for sample D (ie. ECDI/MTX) gave one large peak corresponding to MTX. Fractions 1 & 2 contained nothing detectable by UV; fraction 3 (a shoulder on the main peak) comprised mainly ECDI and fraction 4 was confirmed to be MTX. (yield 99%).

The resolution (Rs) of the peaks was calculated using the following formula:

$$Rs = 2 \times (t_{r2} - t_{r1})/(w_2 + w_1)$$

where t_r is the retention time and w the width of the peak at baseline.

The following values for resolution were obtained:

Rs	HSA/ECDI	=	3.5
Rs	HSA/MTX	=	2.9
Rs	ECDI/MTX	=	1.0
Rs	Conj/ECDI	=	2.8
Rs	Conj/MTX	=	2.6

A resolution value exceeding 1.25 indicates that baseline separation has

been achieved; the above results thus confirmed that the Sephadex fractionation system was capable efficiently of separating HSA or conjugated MTX-HSA from ECDI and free MTX.

2.2.5 Typical synthesis and isolation of MTX-HSA

A typical conjugation reaction was performed as follows:

The reactants were weighed directly into clean, dry volumetric flasks and made up to volume using phosphate-buffered saline (pH7.4, I = 0.29). The required volumes of each solution were then placed in a clean, dry reaction vessel using Gilson automatic pipettes. The sealed vessel was then swirled to mix (Vortex Shaker) and then left in the dark in a water bath at 25°C ± 1°C for 24 hours (Table 2.4).

Table 2.4 Components of a typical conjugation reaction

Substance	Concentration (mg/ml)	Volume added (ml)	Molar ratio
HSA	20.0	3.0	1
ECDI	50.0	0.6	173
MTX	20.2 (as free acid)	2.4	118

The final concentration of HSA in the reaction mixture was 10mg/ml Fractionation of the reaction mix was performed under ambient conditions

using a GPC column which had been pre-equilibrated with mobile phase. The conjugated material eluting with the void volume was collected in a clean dry volumetric flask and made up to volume prior to assay.

Chromatographic conditions:

Column:	Sephadex G15, 1.6 x 68.5 cm
Mobile phase:	PBS, pH 7.4, I = 0.29 (0.45um filtered)
Flow rate:	3.65 ml/min
Detection:	UV 254nm
Injection volume:	5.13 ml
Retention MTX-HSA:	10.4 - 21.25 min
Volume collected:	39.6 ml (then made up to 100ml with PBS)

The collected conjugate solution was then assayed by UV at 376nm following a 1 in 2 dilution with PBS.

The molar conjugation ratio (MCR) was then calculated from the following data.

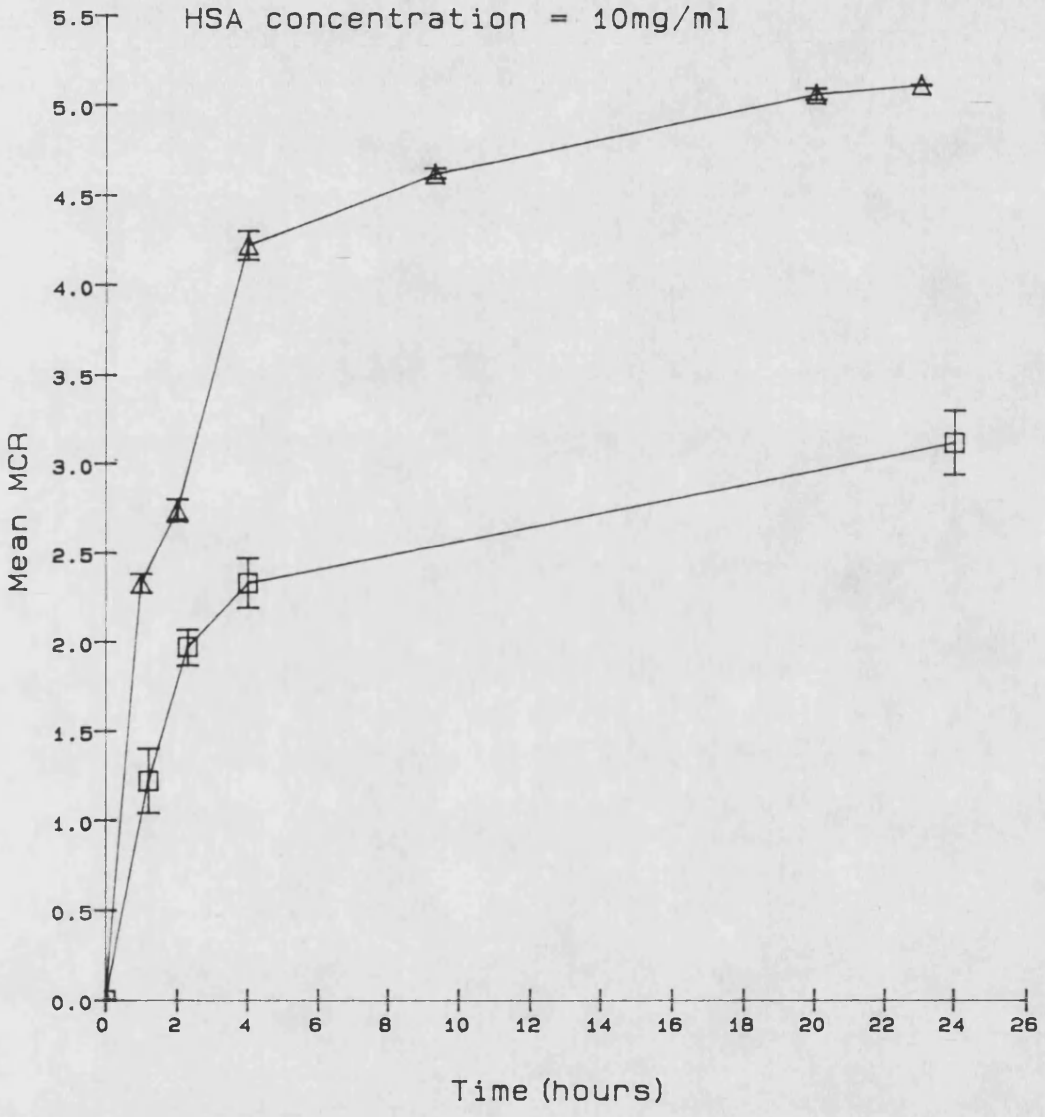
Final HSA concentration (MW 66,241) = 51.34 mg in 100ml Absorbance due to MTX (MW 454.4) at 373nm = 0.598 (E1%/1cm = 162.4)

Therefore, $$MCR = \frac{\text{absorbance} \times 2 \times 1000 \times 66241}{162.4 \times 454.4 \times 51.34}$$

MCR = 20.9 moles MTX per mole HSA

The sample was diluted prior to UV assay only at higher MCR's and

Fig.2.8 Effect of reaction time on the MCR of MTX-HSA conjugates at different temperatures



sufficient determinations were made until a reproducible reading was obtained (I ± 0.002); typically 3 readings.

Batches of lyophilised HSA obtained from Sigma contained water estimated at 5% (Mitsubishi moisture meter). Determination of water content was not routinely available therefore no attempt has been made to correct for the water content of the lyophilised protein.

2.2.6 The effect of reaction time on the molar conjugation ratio

2.2.6.1 Under ambient conditions (18-20°C, dropping to 13-15°C overnight

The reaction was performed as outlined in section 2.2.3 with an HSA :MTX : ECDI ratio of 1:29.2:43.2 (HSA concentration = 10 mg/ml) at a measured pH of 7.45 and left at ambient conditions. 1ml aliquots were removed at different times and fractionated as previously described; each determination was performed at least in duplicate. Results are plotted in Figure 2.8 and tabulated in Appendix 8 (Table A8.2.1)

2.2.6.2 Controlled temperature

The reaction mixtures at pH7.35 (HSA:MTX:ECDI = 1:58.3:43.2 (and 1:29.2:43.2), HSA concentration = 10 mg/ml) were set up in a water bath at 25°C ± 0.5°C and fractionated after various storage times. The results (Figure 2.8, Table A8.2.2) demonstrated that the coupling reaction had virtually ceased after 24 hours. The initial rapid rate of linkage tailed off after 4 hours and the reaction had proceeded to 90% completion

after 9 hours (at 25°C). It was therefore decided to allow subsequent conjugation reactions to proceed overnight, ie. for 12-24 hours, at 25°C prior to termination by GPC.

2.2.7 The effect of the molar ratio of MTX in the reaction mixture on the molar conjugation ratio

The reaction was performed using a fixed mass of ECDI but adding varying amounts of MTX solution and buffer to yield the desired molar ratios. The reaction was carried out overnight at 25°C and a measured pH of 7.35, HSA 10mg/ml, as previously described. Results are plotted in Figure 2.9 and tabulated in Appendix A8 (Table A8.2.3)

2.2.8 The effect of the molar ratio of ECDI in the reaction mixture on the molar conjugation ratio

The experimental procedure in this case was as for experiment 2.2.7 — except that the mass of MTX was constant (except in the absence of ECDI) and the amount of ECDI was varied. Data is plotted in Figure 2.10 and shown in Table A8.2.4

2.2.9 The effect of increasing the concentration of both MTX and ECDI in the reaction mixture relative to that of HSA

The reaction mixtures were set up as before with the exception that the concentration of MTX and ECDI were both increased whilst maintaining a MTX:ECDI ratio of 1:1.5 with HSA dissolved at 10mg/ml. Data are plotted

Fig.2.9 Effect of MTX concentration on the MCR of MTX-HSA conjugates (25°C, PBS pH7.35)

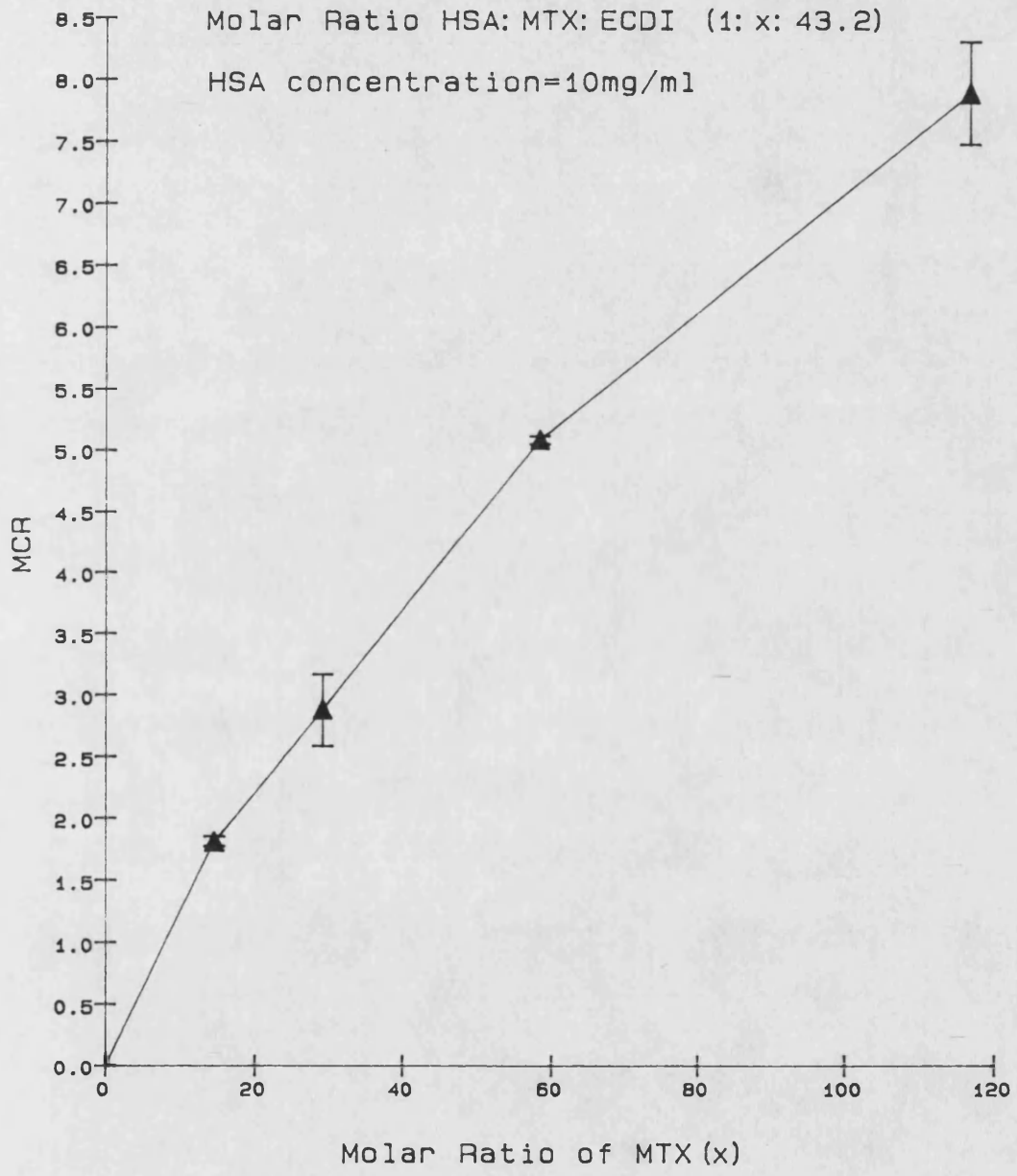
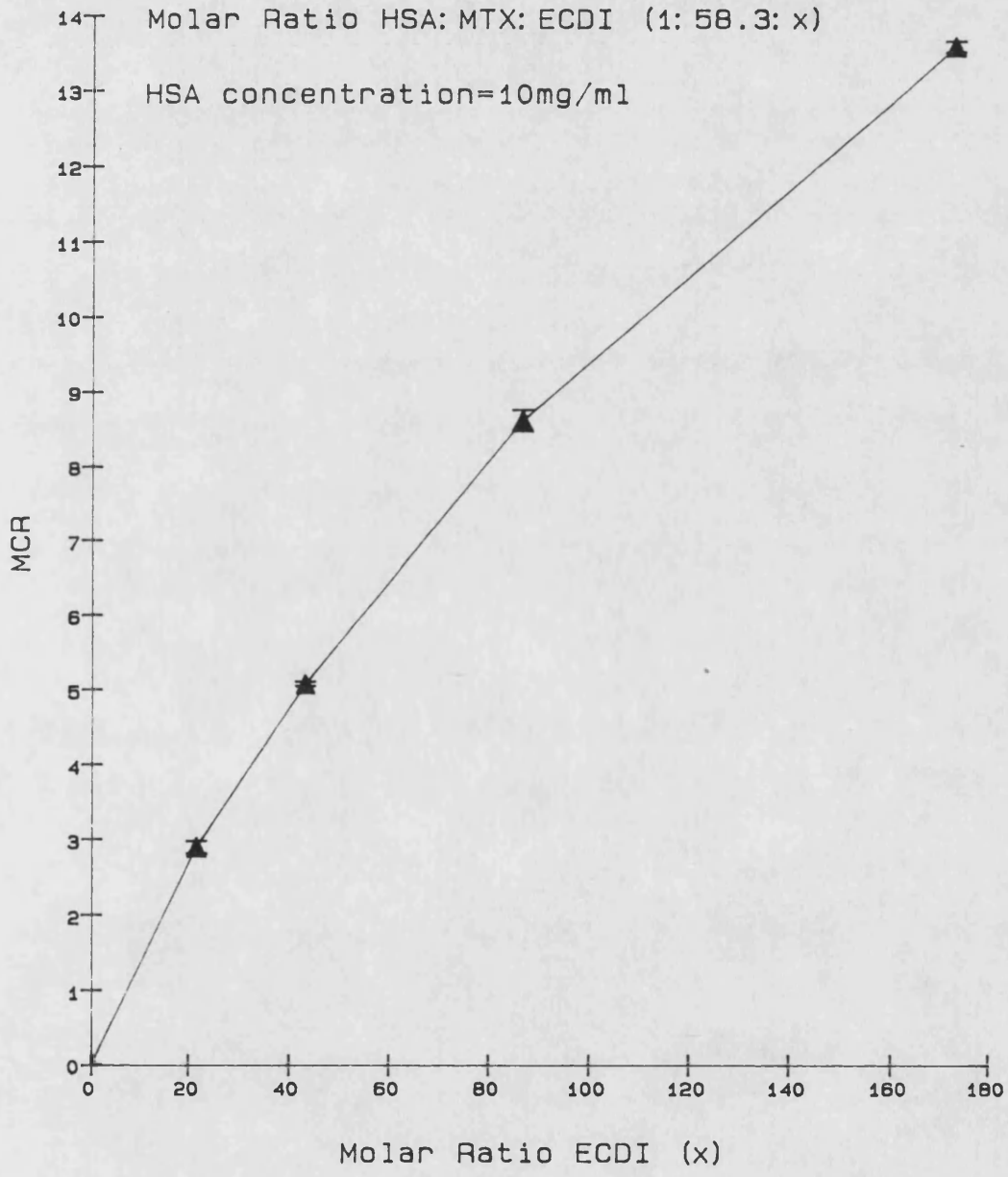


Fig.2.10 Effect of ECDI concentration on the MCR of MTX-HSA conjugates (25°C, PBS pH7.35)



in Figure 2.11 and tabulated in Appendix A8 (Table A8.2.5).

The results from this series of experiments (2.2.7 - 2.2.9) indicated that the MCR was dependent upon the concentrations of both MTX and ECDI. Figures 2.9 - 2.11 demonstrate that as the concentrations of either MTX or ECDI were increased (relative to each other) the conjugation efficiency was reduced, presumably due to an insufficiency of the reactant present at the lower concentration. This was to be expected since the carbodiimide reaction was thought to proceed via the O-acylisourea formed between ECDI and MTX. The curves tended to be linear at lower concentrations, deviating from linearity at higher concentrations.

2.2.10 The effect of pH on the MCR of MTX-HSA

In this experiment the reaction mixtures were produced as before (HSA:MTX:ECDI = 1:58.3:86.4, HSA at 10mg/ml) except that the reactants were initially dissolved in distilled water and the appropriate quantity of double strength phosphate buffer was added to yield final reaction mixtures with the following theoretical pH values: 5.76, 6.12, 6.35, 6.54, 6.72, 6.89, 7.40, 8.10. Ionic strengths was constant at 0.232M. The results (Figure 2.12, Table A8.2.6) are means of two determinations. Figure 2.12 shows that the MCR was reduced with increasing pH between pH 6 and 7 with a subsequent flat portion of the curve. It might be expected that the MCR would increase with a further rise in the pH, as the pKa of lysine -NH_2 is approached, yielding more free amine groups. The improved efficiency at low pH was thought to be due to the fact that the reaction is acid catalysed (the first step being carbodiimide

Fig.2.11 Effect of the concentration of MTX and ECDI on the MCR of MTX-HSA conjugates (25°C, PBS pH7.35)

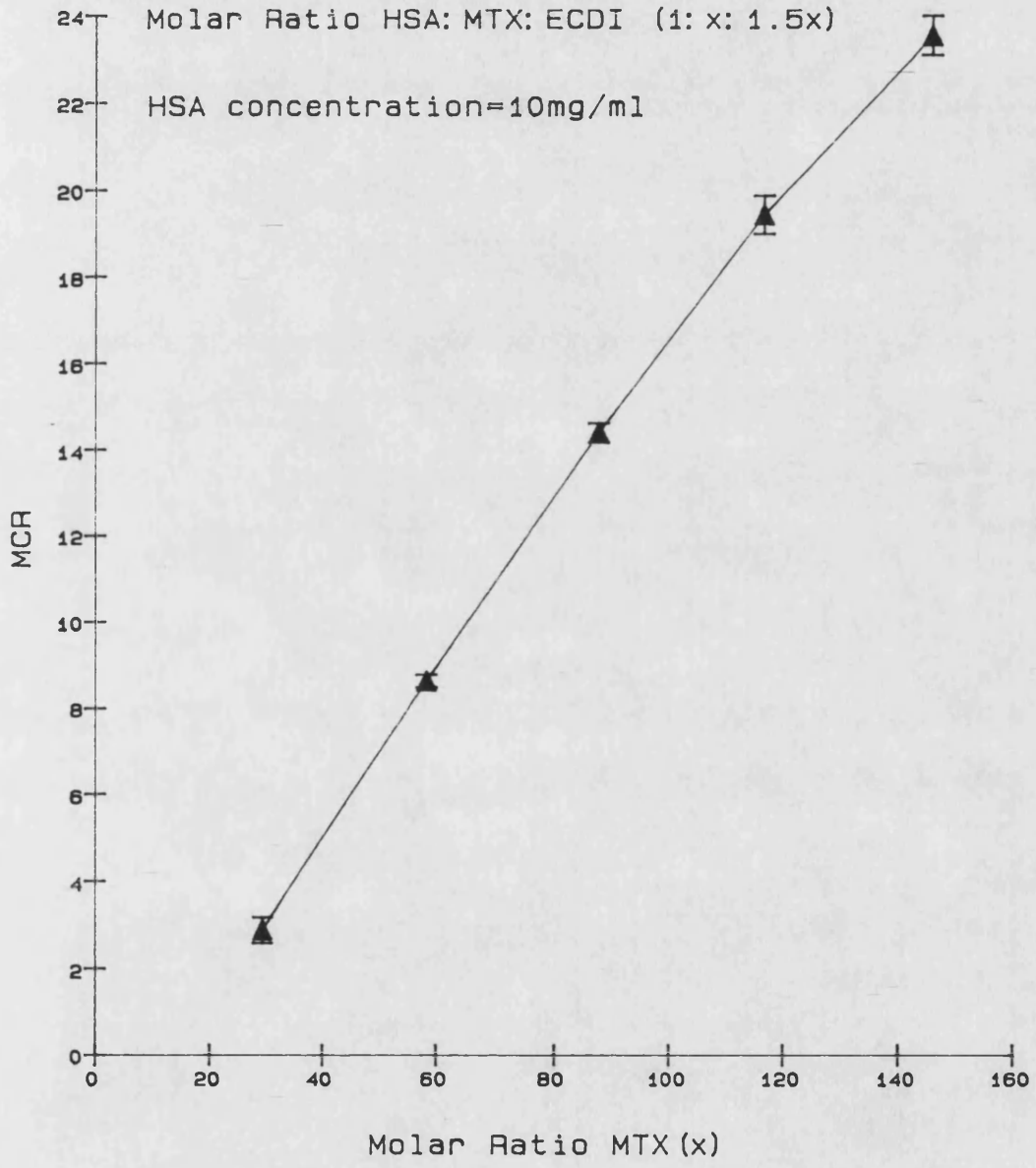
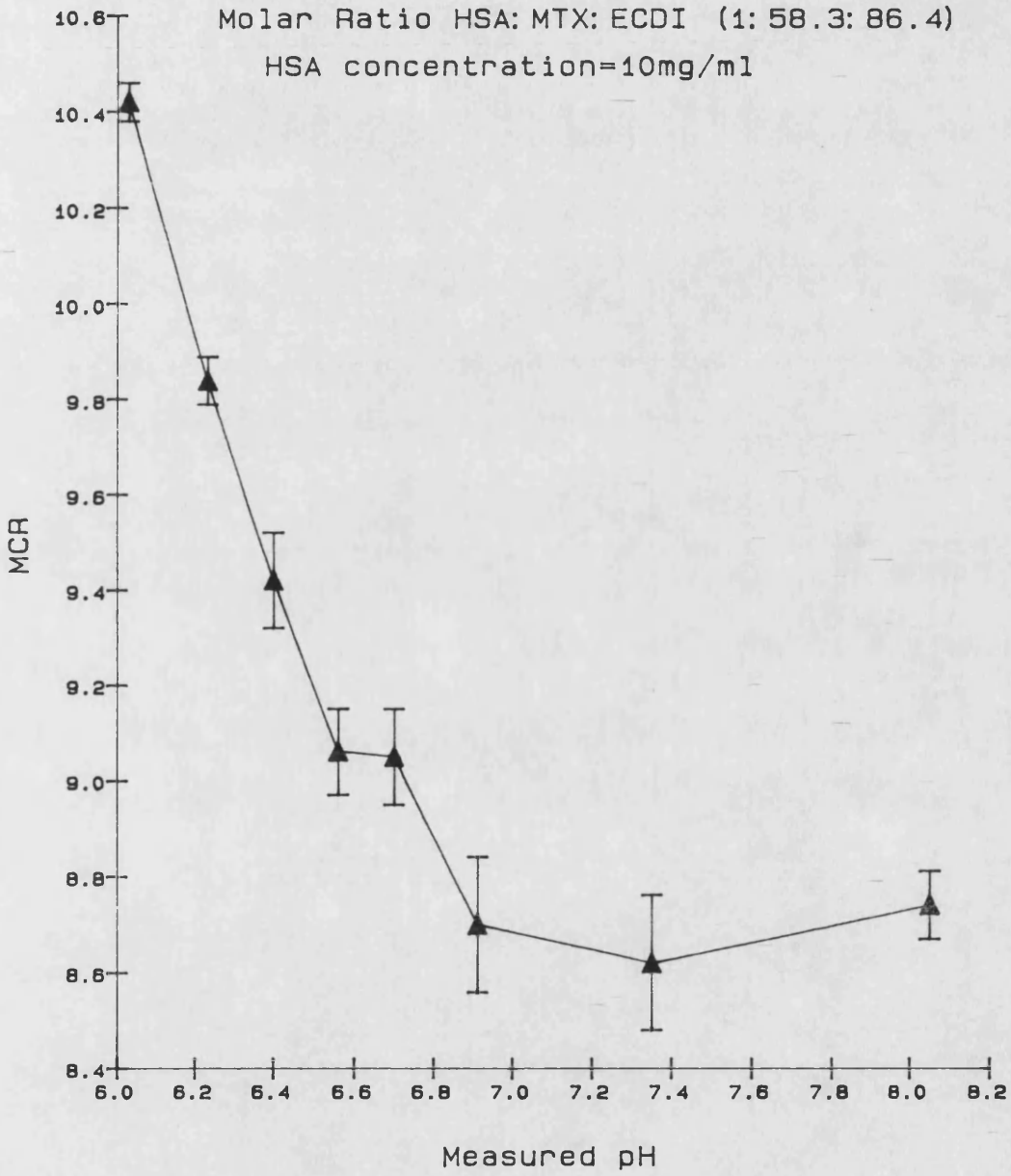


Fig.2.12 Effect of pH on the MCR of MTX-HSA conjugates (25°C)



protonation). Further experiments were conducted at lower pH employing citrate buffers however problems were encountered due to:

i) visible precipitation of MTX below pH5 (ie. as the pKa of COOH was approached).

ii) possible interaction between the carbodiimide and citric acid.

The overall results of the latter experiments were conjugation ratios of less than 1.5 at pH values below 5.9.

Since the intention was to produce conjugates with a reasonable degree of reproducibility and predictability it was decided to conduct all further experiments within the flat region of the curve. This had the advantage that any slight changes in the reaction pH would not have significantly affected the reaction efficiency. To this end a phosphate buffer of pH 7.4 was selected.

2.2.11 Conjugation of MTX to HSA via the active ester

This method involved two steps; the first being the preparation and isolation of the active ester of MTX in a non-aqueous environment, followed secondly by its reaction with HSA in an aqueous environment.

Synthesis of active ester solution

In a typical reaction the following were mixed:-

1. 45.4mg of MTX (as free acid) in 1ml DMF (100 micromoles)
2. 11.5mg N-hydroxysuccinimide in 0.5ml DMF (100 micromoles)
3. 20.6g of DCC in 0.5ml DMF (100 micromoles)

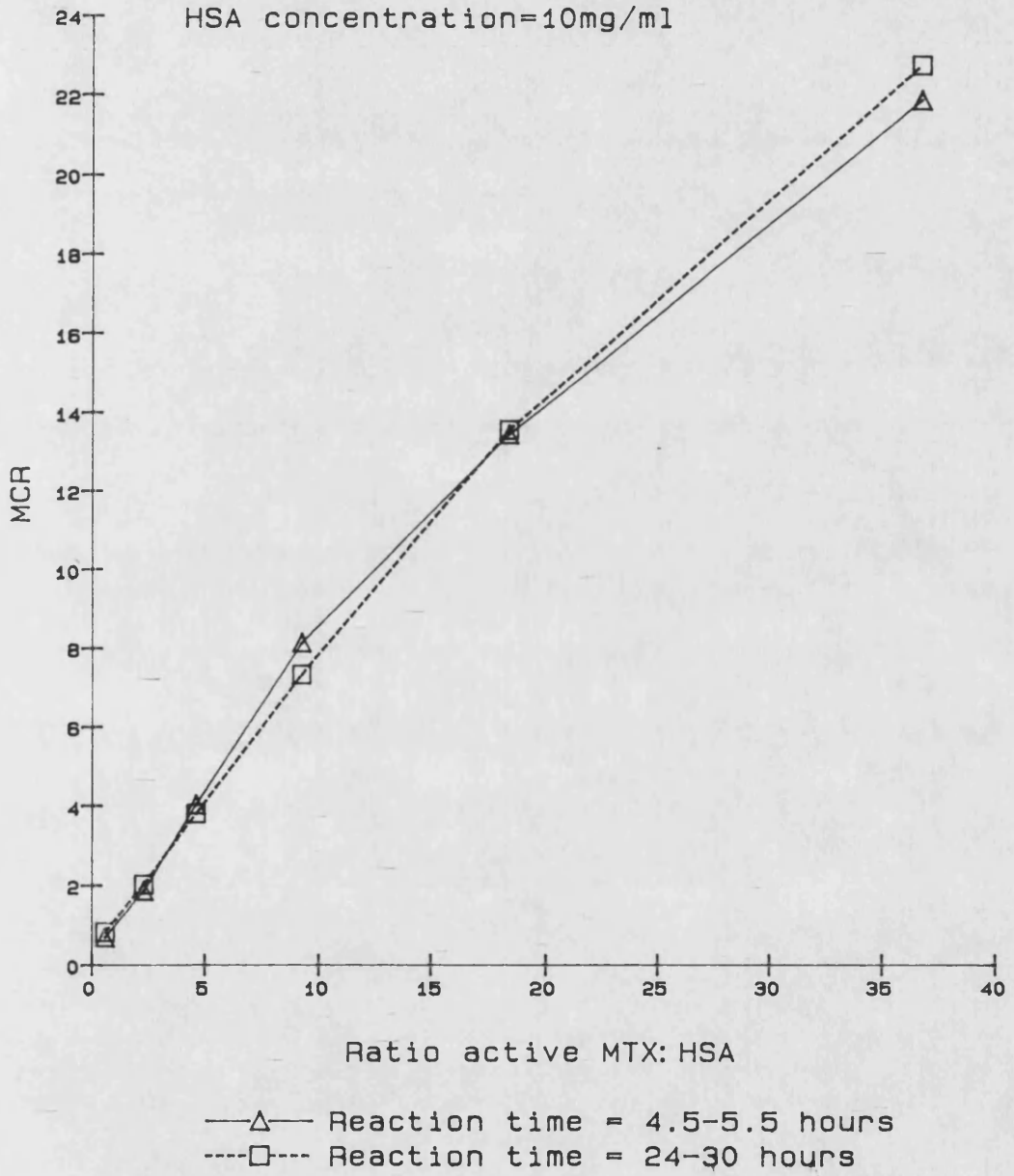
The mixture was stirred at room temperature for 1 hour and then left at 4°C for 18 hours in the dark (under anhydrous conditions). The precipitate which formed was removed either by filtration or removal of the supernatant which was then sealed in vials and stored at 4°C prior to use. Affigel 102 (Bio-Rad) binding experiments indicated that the solution contained about 50% active ester after two months storage; initial experiments on fresh samples showed about 60% active ester, the remainder being free MTX.

Conjugation of activated MTX to HSA

30mg of HSA were dissolved in 2ml of PBS. The appropriate quantities of active ester solution (in DMF) and DMF were added to produce a total volume of 3ml. The samples were left for 4 hours or overnight prior to separation. 1ml aliquots were fractionated on a Sephadex column using PBS as the eluant, the conjugate fraction being collected in 25ml containers. The chromatogram from the separation revealed a second peak eluting close to the high molecular weight conjugate peak; this was confirmed by UV analysis to be DMF. The conjugate fractions were assayed by UV to determine the MCR as described previously.

MCR's were calculated assuming 100% yield of HSA from the Sephadex column ie. HSA = 6.28×10^{-6}M (1.04ml of a 10mg/ml solution diluted to 25ml). Figure 2.13 (see also Table A8.2.7) shows the relationship between MCR and concentration of activated MTX in the reaction mixture.

Fig.2.13 Effect of active ester ratio on the MCR of MTX-HSA conjugates (25°C, PBS pH7.4)



2.2.12 Discussion

The conjugation of MTX to soluble macromolecular carriers (HSA and synthetic polypeptides) has been achieved using a simple one step procedure employing ECDI to create an amide bond between the carboxylic acid residue on the drug and an amine group on the carrier. This reaction has been documented previously with separation of product and reactants being achieved by GPC and dialysis (Halbert, 1983 and 1987). In this study GPC was used extensively to isolate the conjugates and to assess their stability in buffer solutions.

Sephadex gels were used to terminate the conjugation reaction by separating the reactants and by-products from the covalently linked drug-carrier conjugate. The grades of gels (G15, G25 and G50) were selected so as to allow the high molecular weight conjugate to be eluted with the void volume (confirmed using blue dextran, MW 2,000,000) and subsequently collected rapidly in a minimal volume. The globular protein exclusion limit of these columns is 1,500 (G15), 5,000 (G25) and 30,000 (G50) (Pharmacia) thus MTX-HSA conjugates with a molecular weight in excess of 65,000 were excluded and eluted with the void volume, being too large to enter the pores. The grades of Sephadex available are coarse, medium, fine and extra fine; the fine grades afforded better resolution but tended to create excessive back pressures, coarse grades could be run at high flow rates but gave poor resolution. The medium grades were found to offer a good compromise between resolution and flow rate for this type of preparatory work.

The calculation of the molar conjugation ratio (MCR) of MTX to HSA of the isolated conjugate relied upon the following:

1. A known amount of macromolecule applied to the column.
2. The yield of macromolecule/conjugate from the column was 100%.
3. The MTX was covalently bound to the macromolecule.
4. The MTX assay was valid when the drug was conjugated.
5. Molecular weight of the macromolecule.

Each of these points will be considered in turn.

1. The calculation of MCR was not corrected to allow for the water content of lyophilised HSA. A moisture determination was carried out on one batch of HSA by an outside laboratory and found to be approximately 5%w/w. A correction for water content in the calculation of MCR was not applied since batch to batch variation was expected and it was anticipated that the water content of a single batch would have changed upon storage. It was not possible to determine water content routinely at Bath therefore a rigorous correction could not be used. The error introduced into the calculation was expected to be approximately 5% on the basis of the data available. The use of calibrated loops ensured that set volumes of reaction mix were applied to the column each time. A possible problem was adsorption of protein to the plastic tubing and meshes but this was not expected to affect results seriously. Indeed tubing may well have been aged since numerous samples were applied using the same tubing loops.

2. It was shown that virtually 100% of HSA (in aqueous solution at normal concentration) applied to a Sephadex column was eluted as determined by UV

spectrophotometry. The experiment was performed on a freshly cleaned column using distilled water as the mobile phase to maximise the likelihood of binding (low ionic strength increases the chance of ionic interactions although it would reduce hydrophobic interactions). A more sensitive assay could have been employed to determine the level of protein using the Lowry method however, since this method is essentially the UV assay of derivatised proteins, similar errors would have been introduced. Moreover in this case the levels of HSA used gave a significantly high absorbance with which to compare sample and standards (Beer-Lambert linear).

Binding of MTX-HSA conjugates to the column was expected to be very low by inference from refractionation experiments where, following a second separation, the MTX concentration was 97.1% $\pm$ 2.9% of that of the first separation indicating that only a small amount of conjugate was retained by the column. Garnett et al (1984) has attempted to determine conjugate binding to a similar column by radiolabelling both HSA and MTX individually but encountered assay errors which were too large to precisely determine the extent of binding.

3. The calculation of MCR assumed that any MTX eluting with the void volume was covalently bound to the HSA and that none of this material was physically adsorbed on to the macromolecule (protein binding). The binding of MTX to albumin is a well documented phenomenon (Steele et al, 1979).

The ability of the Sephadex column to separate high molecular weight molecules from low molecular weight material (ECDI and MTX) was

demonstrated with baseline resolution being achieved between HSA/MTX-HSA and MTX/ECDI derivatives. The high molecular weight material was eluted with the void volume (Kav = 0) with minimal sample dilution. The ECDI derivative was eluted with a Kav = 0.8 as a shoulder on the broad MTX peak (Kav = 1.2), the high Kav value of the latter peak infers that a degree of column interaction occurred. This could have been due to either ionic interactions of the ionised carboxylic acid residues (however this should be minimised by the high ionic strength of the mobile phase) or hydrophobic interactions involving the aromatic rings on the drug molecule. The covalent linking of MTX to Sephadex (mediated by ECDI) was unlikely since ECDI is inactivated in aqueous solution and following a reaction time in excess of 12 hours would only have had a limited ability to cause either cross linking of the gel or binding of MTX to the gel. In support of this view it was observed that samples which omitted either HSA or ECDI still gave the same Kav for MTX suggesting that the interaction was not covalent in nature; this was confirmed by the 100% yield of MTX from the column over a reasonable time scale. A covalent link would have been slowly hydrolysed under the relevant conditions of pH and temperature. The lack of physical binding of MTX to HSA was confirmed by UV analysis of HSA following incubation with MTX and separation on a Sephadex column. The spectrum of the high molecular weight fraction was not significantly different from that of the standard HSA solution. The absence of MTX in this sample was confirmed by FPLC analysis showing little difference between peak areas of the separated HSA sample and the standard, neither exhibiting the presence of free MTX either initially nor after incubation at 37°C for 24 hours.

4. The assay to calculate MCR involves determination of the MTX concentration using its E1%/1cm at its λ max at 370nm. However a bathochromic shift occurs altering the λ max to 376nm. This phenomenon is not observed in binary mixtures of HSA and MTX (although the spectrum is essentially the same) which also implied that the linkage between the HSA and MTX is covalent in nature. It is assumed that the use of E1%/1cm at 370nm is valid in calculating the MTX concentration since the peak is fairly broad and induced errors would be minimal (see Appendix A6).

5. The molecular weight of HSA has been variously reported to be between 66,000 and 69,000 however the value used in these calculations is determined from its composition as given in the Atlas of Protein Sequence and Structure ie. 66,241. The conjugation reaction proceeded to virtual completion after 24 hours at 25°C (from MCR calculations) with approximately 80% of this value being reached after 4 hours. Higher activities of MTX or an increase in temperature resulted in faster initial rates of reaction. The inclusion of temperature control during the reaction yielded MCR with lower standard deviations implying a more controlled reaction. The slowing of the reaction is due to the consumption of one of the reactants; this is unlikely to be the MTX since it is generally present at a large excess (linking efficiency in this case being around 10% ie number of moles of MTX conjugated expressed as percentage of the number of moles in the reaction mixture). The drop in rate could either be due to all the accessible linkage sites on HSA being occupied (however increased levels of MTX/ECDI result in higher MCR's) or the ECDI being consumed either by reaction with MTX/HSA or the buffer solution. It has been demonstrated that ECDI solutions (in PBS pH7.4) stored overnight (2-8°C) resulted in MCR's only 13% of those produced

using fresh ECDI solutions. This suggests that the drop in reaction rate is due to the ECDI reacting with water. As a consequence of this the buffer solution was added to the dry ECDI reagent immediately before aliquots were added to the mixtures of HSA and MTX solutions. Higher reaction efficiency could be achieved by adding the HSA/MTX solution to the dry carbodiimide reagent, however this technique proved less accurate due to the small amounts (by weight) of the reagent involved.

The effect of varying the level of MTX (HSA and ECDI constant) showed that increasing the level of MTX resulted in an increase in the MCR; this was however accompanied by a drop in MTX linking efficiency from 12.4% (HSA:MTX = 14.6) to 6.8% (at 116.5). A similar experiment varying the relative level of ECDI (HSA:MTX = 1:58.3:x) gave the expected increase in MCR with an associated increase in MTX linking efficiency from 5.0% (at HSA:ECDI = 21.6) to 23.3.% (at 173.0). The graphs of both experiments (Figures 2.9 and 2.10) exhibited a curvilinear relationship suggesting that the reaction can be limited by levels of either reactants. The increasing efficiency with increasing amount of ECDI implies that this had more influence on the resultant MCR than did MTX, provided a certain level of MTX was present. A more linear relationship was obtained when the ratio of MTX:ECDI was kept constant and their concentrations relative to HSA were increased. The MTX linking efficiency increased from 10 to 15% as the amount of MTX was increased but stabilised at 16-17% for the three higher levels. The line fitted to the points tails off at higher levels (Figure 2.11) suggesting that there are a limited number of binding sites on HSA. If it is assumed that MTX only binds to lysine residues then the maximum MCR is 59. It is unlikely that all these residues will be accessible although they will tend to be located on the surface of the

molecule due to their hydrophilic nature. The regression line (r = 0.997) does not pass through the origin (intercepts the x axis at c.10 molar ratio MTX) it may be concluded from this that a certain level of MTX is necessary otherwise side reactions could predominate (ie with H_2O or HSA).

The conjugation reaction when conducted in a range of phosphate buffers (pH.6-8) showed that a plateau region between pH.6.9 and 8.1 (ie no significant difference between the MCRs) corresponding to minimum efficiency (15%). At lower pH the MCR increased (as did the efficiency) however this was limited by precipitation of the product below pH6 (and interaction with the buffer). The increase in linking efficiency at lower pH may be accounted for by the acid catalysis of the carbodiimide reaction. It is also suggested that the MCR may increase once more at high pH as the pKa of lysine is approached thus increasing the number of unprotonated residues (Garnett et al, 1984).

This series of experiments indicates that provided the temperature and pH of the reaction mixture are controlled and that a freshly prepared solution of ECDI is used then a MTX-HSA conjugate of a particular MCR can be prepared by selecting the appropriate proportions of each reactant.

The linking of MTX to HSA via the activated ester provided a much more efficient reaction with 30-45% of the input MTX being conjugated to HSA. The conjugation had reached 90-100% of the 24 hour MCR value after 5.5 hours. The separation of the conjugate from the MTX/ECDI derivative is as efficient as previously observed for carbodiimide-mediated reaction however it was noted that the separation of the conjugate from the DMF was not as good as might have been expected leading to possible contamination

of the conjugate solution by the solvent.

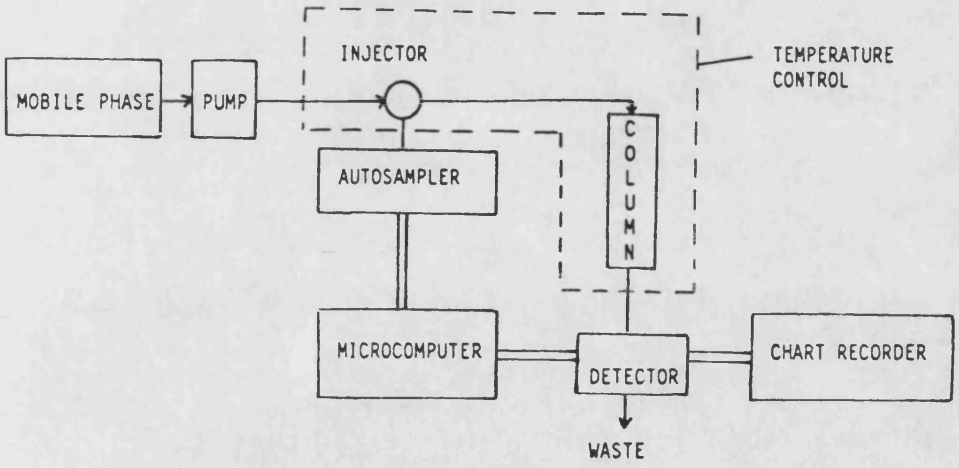
The active ester method of conjugation has the advantage over the ECDI reaction in that it is more efficient, however the procedure is more complex in that it involves two steps and an intermediate assay. The presence of DMF may also present a separation problem as described above and also affects the solubility of HSA limiting the ultimate degree of conjugation.

2.3 FPLC analysis of MTX-HSA conjugates

2.3.1 Introduction

Halbert et al (1983) demonstrated using dialysis experiments that MTX-BSA conjugates were unstable in buffer solutions. It was decided in this study to assess the stability of MTX-HSA conjugates using fast protein liquid chromatography (FPLC). This technique allows rapid, high resolution GPC enabling analytical separation of materials according to molecular weight. The analytical system (similar to that of a conventional HPLC) was as follows (Figure 2.14):

Figure 2.14 Schematic diagram of FPLC system



Mobile phase:	as used for isolation of MTX-HSA
	PBS pH 7.4, I = 0.29M, filtered (0.45um), degassed
Pump:	LDC Constametric II with pressure monitor
Injector:	Rheodyne 7132
Autosampler:	ACS
Column:	Superose 12 (Pharmacia) or TSK G3000PWxl
Detector:	Gilson 113
Chart recorder:	BBC Servogor 212, dual pen
Microcomputer:	Acorn BBC Master linked to Winchester hard disc via an Econet network

2.3.2 Sample preparation and application

The conjugates were prepared as previously outlined (section 2.2.5) and then desalted on a Sephadex GPC column. The samples were then filtered (sterile 0.2um) into sterile vials for storage under specified conditions. Samples were applied to FPLC columns using one of the following methods:

i) multiple sampling from a solution held at 37°C using a modified autosampler injection valve.

ii) samples removed from 37°C storage and injected using the autosampler.

iii) samples injected manually using the autosampler to trigger data capture (by the microcomputer).

Samples stored in vials for methods ii) and iii) were either applied immediately upon removal from 37°C storage or held at -18°C prior to assay. The volume of sample applied depended upon the column ie. 200 microlitres for Superose 12 and 20 microlitres for TSK.

2.3.3 Columns

The columns used were either

i) Superose 12 HR 10/30 10mm x 30 cm which contained cross-linked agarose beads of diameter 10 ± 2 um. This was suitable for proteins of molecular weight up to 300,000 daltons.

ii) TSK G3000PWxL 7.8mm x 30cm
+ TSK guard column PWH 7.5mm x 7.5cm

The flow rates were selected to enable rapid analysis whilst still providing good resolution

Column	Flow Rate	Analysis time
Superose 12	0.5ml/min	60 min
TSK G3000PWxl	0.75ml/min	30 min

2.3.4 Temperature control

The temperature of the column was maintained at 25°C ± 0.5°C by either immersing the column in a water bath (TSK) or using a column jacket (Superose). A heat exchanging coil was used to control the temperature of the mobile phase and the valve/loop was also immersed in the water bath.

2.3.5 Detection and data capture

The detectors used were a Pye Unicam UV20 (fixed wavelength 254 & 280), Pye Unicam UV3 (variable wavelength), LDC Spectromonitor (variable) and, more routinely, a Gilson 113. The Gilson 113 was a variable wavelength detector which could be used as a single wavelength detector with two outputs at different sensitivities or a dual wavelength detector and could also be used to scan peaks of particular interest. The two outputs were monitored using a dual channel chart recorder; one of the outputs also being digitised using an Acorn BBC computer. Data was logged on to a Winchester hard disc for subsequent analysis; to generate information on retention times, peak areas and for computation of molecular weight distributions. The autosampler was used remotely to trigger the computer data capture system thus enabling retention times to be calculated more precisely.

2.3.6 Column calibration

It was intended to use the FPLC system to investigate the stability of the

conjugates, release of free MTX and also to study the effects of enzymes on the carrier system. Therefore it was necessary to calibrate the columns with proteins of known molecular weight to enable the molecular weight distributions to be determined and also to calibrate with MTX and HSA in order to quantify the release of MTX from the conjugates. Details of calibration procedures and the data obtained are described in Appendix A2. In summary both columns could be calibrated for molecular weight in accordance with theory. When log molecular weight was plotted against Kav both columns gave a linear fit over a limited range. The Superose column was effective over a wider molecular weight range (1 - 100 kD) than the TSK G3000 column (2 - 70 kD) but the latter column was more robust and less variable in day to day use. The Superose column compressed during use such that its total volume varied during a series of assays. Regular calibration was thus necessary to obtain accurate data but this disadvantage was outweighed by its superior resolution, particularly of HSA and aggregates of HSA. MTX interacted weakly with both columns which resulted in Kav values slightly greater than unity (Kav MTX was 1.2 and 1.1 on the Superose and TSK columns respectively). Both columns could be calibrated for MTX or HSA concentrations giving linear plots against peak height or peak area over the relevant concentration range studied.

2.3.7 Resolution of HSA and MTX by FPLC columns

2.3.7.1 The ability of the Superose 12 column to separate HSA and MTX

The purpose of this experiment was to determine whether or not the Superose 12 column was capable of separating MTX from HSA under routine operating conditions.

Samples were prepared by taking aliquots from stock solutions of HSA and MTX to obtain a range of MTX and HSA concentrations which were equivalent to those present in conjugates following synthesis and fractionation (molar ratios of MTX:HSA 0.03 to 21.3) Samples were then placed in vials and injected soon after preparation using the autosampler. Other samples were stored at ambient temperature and injected after a period of at least 12 hours storage. Chromatograms were compared with those of HSA and MTX standards to calculate the relative concentration either by peak height or area (CI10 integration) at 300nm. Data obtained is shown in Table 2.5. The results from this experiment indicated that Superose 12 chromatography could be used to analyse both HSA and MTX in the same sample, good correlations being achieved in both calibration exercises. The system was capable of separating physical mixtures of HSA and MTX with good baseline separation being achieved at all concentrations studied (Rs > 2.4). The separated HSA fraction did not contain any physically bound MTX since at 300nm this would have had a significant affect on UV absorption. Statistical analysis (t test) showed that the standard and test sample peak heights of HSA were not significantly different. The mean retention times were 25.3 ($\pm$ 0.1)min and 49.6 ($\pm$0.4)min for HSA and MTX respectively.

2.3.7.2 The ability of the TSK column to separate HSA and MTX

Samples were prepared from stock solutions of HSA (5mg/ml) and MTX (0.137mg/ml) in PBS pH7.4. The required amounts were mixed and made up to volume with PBS to produce the following molar ratios (MTX:HSA) 1,5,10 and 20. MTX and HSA standard solutions were also prepared with the same concentrations as the mixed samples. Samples were stored at ambient

Table 2.5:
The separation of physical mixtures of MTX and HSA using the Superose 12 column (detection at 300 nm) (HSA = 0.513 mg/ml) (PBS, pH 7.4, 25°C, 0.5 ml/min)

Sample	Molar ratio MTX : 1 HSA	Rs	HSA % of input by peak height	HSA % of input by peak area	MTX % of input by peak height	MTX % of input by peak area	MTX % of standard by peak height
RM13/1	0.028	5.5	99.2 (±0.5)	99.7 (±0.4)	100.5 (±0.5)	poor integration	-
RM13/2	0.057	4.9	99.0 (±0.3)	99.1 (±0.1)	99.3 (±0.3)	poor integration	-
RM13/3	0.085	4.5	100.0 (±0.3)	101.1 (±1.5)	98.5 (±0.2)	poor integration	-
RM13/4	0.114	4.3	99.9 (±0.2)	97.8	99.7 (±0.7)	99.4 (±1.9)	-
RM13/5	0.142	4.1	99.6 (±0.2)	99.5 (±0.2)	100.2 (±0.2)	102.1 (±0.3)	-
RM15 C	0.85	3.5	101.8 (±0.5)	101.2	99.5 (±2.5)	101.0	100.7
RM15 B	5.4	2.7	102.1 (±0.8)	99.1	100.3 (±4.0)	97.9	102.2
RM15 A	21.3	2.4	101.4 (±1.1)	100.7 (±0.2)	99.2	99.9	104.1

(±sd)

temperature (for up to 30 hours) and then injected onto the TSK column with detection at 279 and 303nm. The mobile phase was PBS pH7.4 at 25°C; flow rate 0.746ml/min. Data is shown in Tables 2.6 and 2.7.

The mean retention times were:

HSA = 11.47 (±0.05)min, Kav = 0.145

MTX = 24.93 (±0.07)min, Kav = 1.090

The resolution values were calculated giving Rs > 3.4 which indicated a good baseline separation. The results showed that the TSK column could be used to assay both MTX and HSA and that it was capable of separating mixtures efficiently (with 100% yield). The baseline resolution was aided by the weak interaction of MTX with the support material.

2.3.8 Discussion

The stability of drug/macromolecular conjugates has previously been examined by way of dialysis experiments (Halbert, 1983). In this study MTX-HSA stability was examined using FPLC which had the potential to assay for free MTX and also check the molecular weight integrity of the carrier system simultaneously. This also overcomes any problems associated with dialysis experiments eg adsorption, microbial contamination, time delay to equilibrium and any inconsistencies due to poor mixing in the bulk phase.

The mobile phase used in the FPLC analyses was prepared as for that used in fractionation experiments ie recently prepared, sterile filtered PBS

Table 2.6:
Separation of physical mixes of HSA and MTX on the TSK column: MTX assay at 303 nm (and 279 nm) (PBS, pH 7.4, 25°C, 0.75 ml/min)

Sample/ Molar ratio MTX:HSA	MTX Concentration mg/ml	303 nm		279 nm
		Sample peak area as % std	Sample peak area as % std	Sample peak height as % std
RM25/1	2.74×10^{-3}	100.8	102.6	100.0
RM25/5	1.37×10^{-2}	99.3	99.8	100.0
RM25/10	2.74×10^{-2}	100.0	99.4	100.0
RM25/20	5.48×10^{-2}	100.0	101.2	100.4

Table 2.7:
Separation of physical mixes of HSA and MTX on the TSK column: HSA assay at 279 nm (HSA = 6.0×10^{-3}M) (PBS, pH 7.4, 25°C, (0.75 ml/min)

Sample	Sample peak height as % std	Sample peak area as % std
RM25/1	100.4	97.8
RM25/5	99.1	98.9
RM25/10	100.7	99.8
RM25/20	101.5	102.0

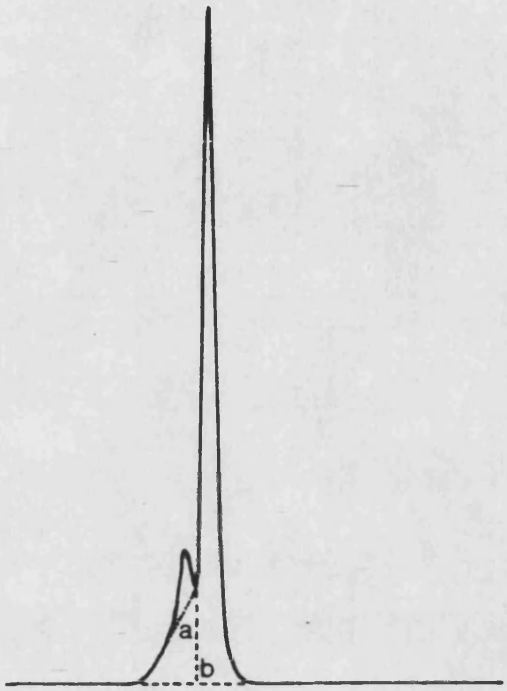
pH7.4. This was to prevent microbial spoilage of the columns since although a low level of contamination was unlikely to alter the conjugate itself, due to the short run times involved, it may have altered the chromatographic characteristics of the separation systems (eg reduce the plate number). In order to minimise the risk of contamination 0.02% w/v sodium azide was incorporated into the mobile phase and the eluant was kept flowing through the column continuously. The high ionic strength was employed to minimise ionic interactions.

The sample preparation was performed under clean laboratory conditions in order to minimise the risk of sample contamination. The Sephadex columns were regularly cleaned according to manufacturers instructions ie with NaOH and 70% ethanol and were left in ethanolic solution if stored for long periods of time. The buffer solutions were recently or freshly prepared filtered 0.2um sterile filter and stored in clean/sterile sealed Duran bottles at 2-8°C. In later experiments 0.02% w/v sodium azide was incorporated into the mobile phase to reduce the probability of microbial spoilage of either the column or conjugate solution. All glassware used in sample preparation and collection was cleaned, dried and foil wrapped before sterilisation in a dry oven at 160°C for a minimum of 2 hours (more routinely overnight). Vial caps were soaked in 70% ethanol solution then dried briefly at 80-100°C. The sample solution following collection from the Sephadex column was immediately sterile filtered (0.2um) into vials. The columns were selected on the basis of their claimed separation range for proteins which in the case of the Superose 12 was up to 300,000 Daltons and for TSK was 100,000 Daltons. The required molecular weight separation range was 1,000-70,000 Daltons. The Superose 12 column had the advantage that it was capable of separating the dimer of HSA but was

however prone to column compression during a run and periodical blocking. The TSK which was slightly inferior in terms of its range of separation but did not suffer from the aforementioned problems and had an analysis time of only 30 minutes (as opposed to 60 minutes for the Superose 12).

The use of temperature control of the FPLC system was possibly not absolutely necessary but helped to prevent baseline drift due to the detector which was observed during overnight analysis. The more controlled conditions also resulted in more consistent retention times due to the mobile phase viscosity remaining constant. The analogue output from the detector was digitised and the data captured by an Acorn BBC microcomputer. The data was stored on a 30Mbyte hard disc, which was necessitated by the large amount of data and subsequently analysed. The analysis of molecular weight programme (see Appendix A7) enabled the precise calculation of retention times. The programme also enabled determination of area under the curve (AUC) and molecular weight distributions (from the MW calibration) either overall or of particular peaks. The analysis of specific peaks could be achieved either by skimming (a) or by dropping a perpendicular to a preset baseline (b) from a particular point (Figure 2.15). The molecular weight distribution being determined by normalisation of the peak area and MW calibration (see section 2.2.2.). The chromatogram (Figure 2.15) shows the smaller dimer peak of HSA (c. 137,000) eluting prior to the larger monomer peak (c. 67,000). These are referred to as the dimer peak and monomer peak.

Figure 2.15 Chromatogram of MTX-HSA conjugate from Superose 12 showing peak area analysis



The molecular weight calibration of the columns was achieved using a Pharmacia protein calibration kit. The Superose 12 gave a good regression line over the MW range 6,500-134,000 however it may be observed that the slope of the lines obtained were not consistent which may arise due to changes in the condition of the column (although it was regularly cleaned according to the manufacturers instructions). In order to ensure accurate results calibration standards were employed during each assay where molecular weights were to be determined (at least at the beginning and end of each run) and these results used in the calculation of molecular weight distributions.

The calibration of the TSK G3000 PWxl column proved more reproducible however calibration standards were employed routinely as for the Superose

column. The regression line fitted to the calibration points from MW 1353 to 67,000 gave a reasonable straight line however it can be seen from Figure A2.5 which includes the BSA dimer that the line is the classical sigmoid shape expected in GPC. It shows that the column is linear between 6,500 and 43,000 implying a useful range between about 5,000-50,000. A non-linear regression line would be more appropriate in this case however since this was unavailable it was thought acceptable to rely upon the linear regression analysis to calculate the molecular weights. It is known that this would give slightly erroneous values in some cases but it was thought the system would still prove valuable since the molecular weights were to be determined over the width of a normalised peak fraction as opposed to a one point determination.

The calibrations with MTX proved linear over a wide range of concentrations at the wavelengths employed on both columns. The method of quantification (peak height or area) had no effect on the fit of the regression line. The retention times were also very reproducible with Kav values at 1.1 for the TSK column and 1.2 for the Superose 12 column suggesting an interaction with the stationary phase in both cases.

The HSA calibration at 279nm for the TSK and 280nm for the Superose 12 both yielded good calibration lines and reproducible retention times. A similar situation was observed with the diluted conjugate solution on the Superose column which showed a linear response for both conjugate and MTX using dilutions of 33.3% to 100%. It is concluded from this that no significant levels of adsorption occur over the range studied since if this were the case then a deviation from linearity may be expected at the lower concentrations. The ability of the columns to separate physical

mixtures of MTX and HSA was demonstrated using a range of MTX:HSA molar ratios from 1 to 20 (and as low as 0.03 in the case of the Superose based system). In both systems complete separation of both components was achieved (ie 100% of standard solutions) with Rs > 2.4 for the Superose and Rs > 3.4 for the TSK. This confirms that any MTX that might be physically bound to HSA would be separated on the first pass through the FPLC column even down to molar ratios of 0.03 moles MTX to 1 mole of HSA. Even at this level of adsorption a 10% increase in peak areas would be observed at 300nm. This is important to confirm that any release that may occur is not due to physically bound MTX.

2.4 The stability of MTX-HSA conjugates in buffer solution

2.4.1 Effect of MCR on stability of MTX-HSA conjugates

The purpose of the experiments described in section 2.4 was to investigate whether MTX-HSA conjugates maintained their integrity when stored in solution at elevated temperature. Preliminary experiments indicated that the conjugates released free MTX upon storage in buffer solutions at 37°C, which confirmed the findings of Halbert (1983).

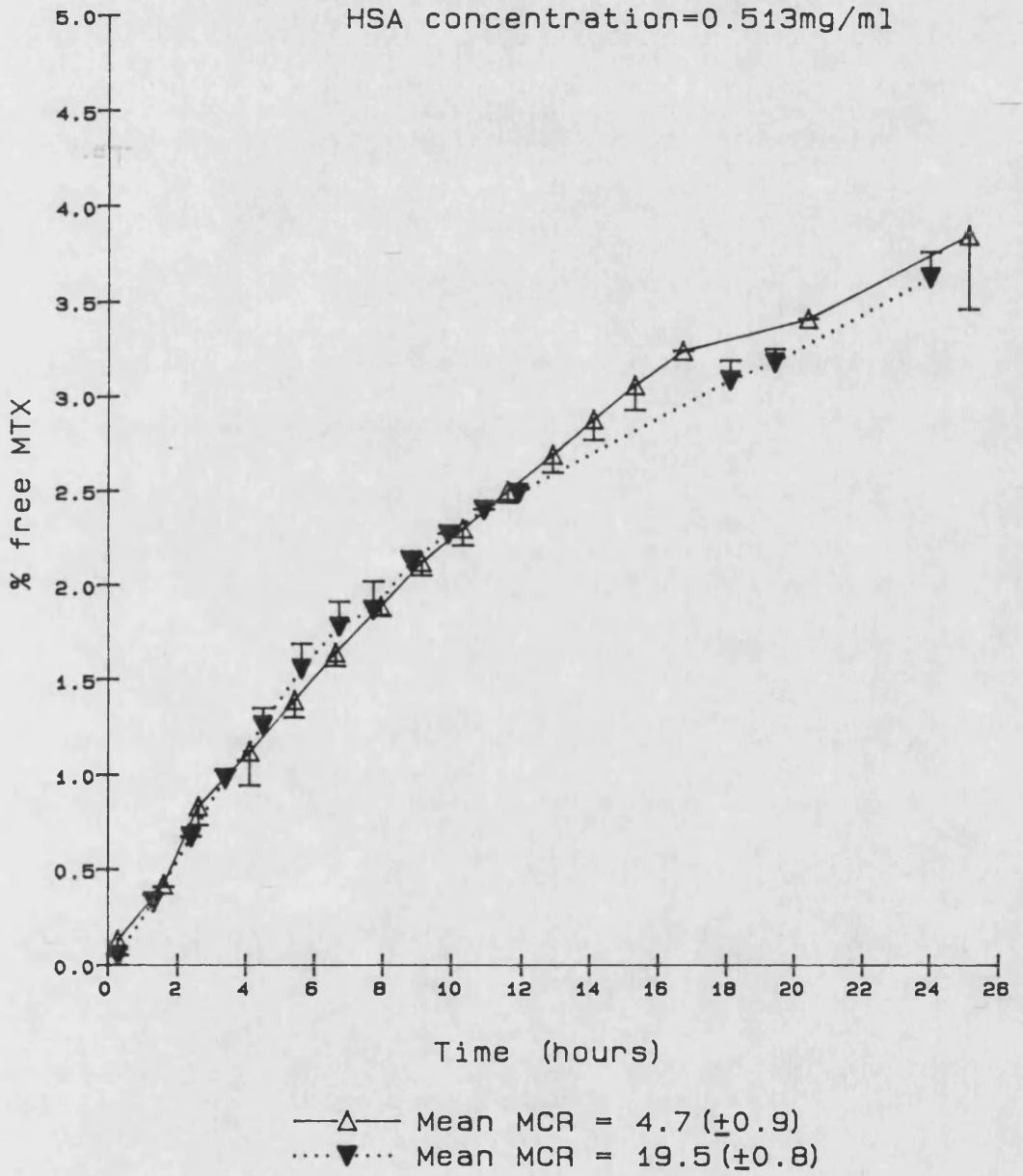
The preparation of the samples was as outlined in section 2.2.5; ie. 24hr conjugation at 25°C followed by Sephadex fractionation (PBS pH7.4 as eluant) and sterile filtration. The sample was repeatedly injected on to the Superose column from a container held at 37°C using the autosampler. The concentration of MTX present in the sample was determined by UV assay at 376nm and the MCR calculated (section 2.2.5). Stability test samples

were interspersed with suitable MTX standards. The amount of free MTX released was determined and expressed as a percentage of the total MTX concentration.

The conjugate peak was observed to elute first with a similar peak shape to HSA alone, the major difference being an increased proportion of dimer in the conjugated sample. Dimer and monomer peak shape did not alter during a 60 hour run indicating that the tertiary structure of the conjugate was unaffected by storage. An example of a MTX-HSA conjugate peak (on the Superose column) is shown in Figure 2.15. Conjugate samples injected immediately following fractionation showed very low levels, or non-detectable amounts, of free MTX. Low levels of free MTX arose due to a limited amount of degradation occurring during sample processing after fractionation, since the first possible sample point was 30 minutes from application to the Sephadex column and 10-15 minutes after emergence of MTX-HSA conjugate. Results obtained for release of MTX from conjugates with low and high MCR's are shown in Table A8.2.8 and plotted in Figure 2.16. Each result was the mean of two separate experiments.

It was concluded from this experiment that the amount of MTX released from the conjugates, when expressed as a percent, was not dependent on the MCR of the carrier system. The amount of cleavable bonds produced by the conjugation reaction was proportional to the MCR.

Fig.2.16 Release of free MTX from MTX-HSA conjugates of different MCR (37 °C, PBS pH7.4)



2.4.2 The effect of the reaction time on conjugate stability

In this experiment the conjugation reactions were performed in the usual manner except that for runs 3, 4 and 5 the ECDI solution had been prepared about 2 hours prior to use, thus resulting in a lower conjugation ratio than would have been expected from the 1: 116.5:43.2 ratios employed. However they could be used for comparative purposes (runs 1 and 2 were produced using fresh ECDI using a 1:58.3:43.2 ratio).

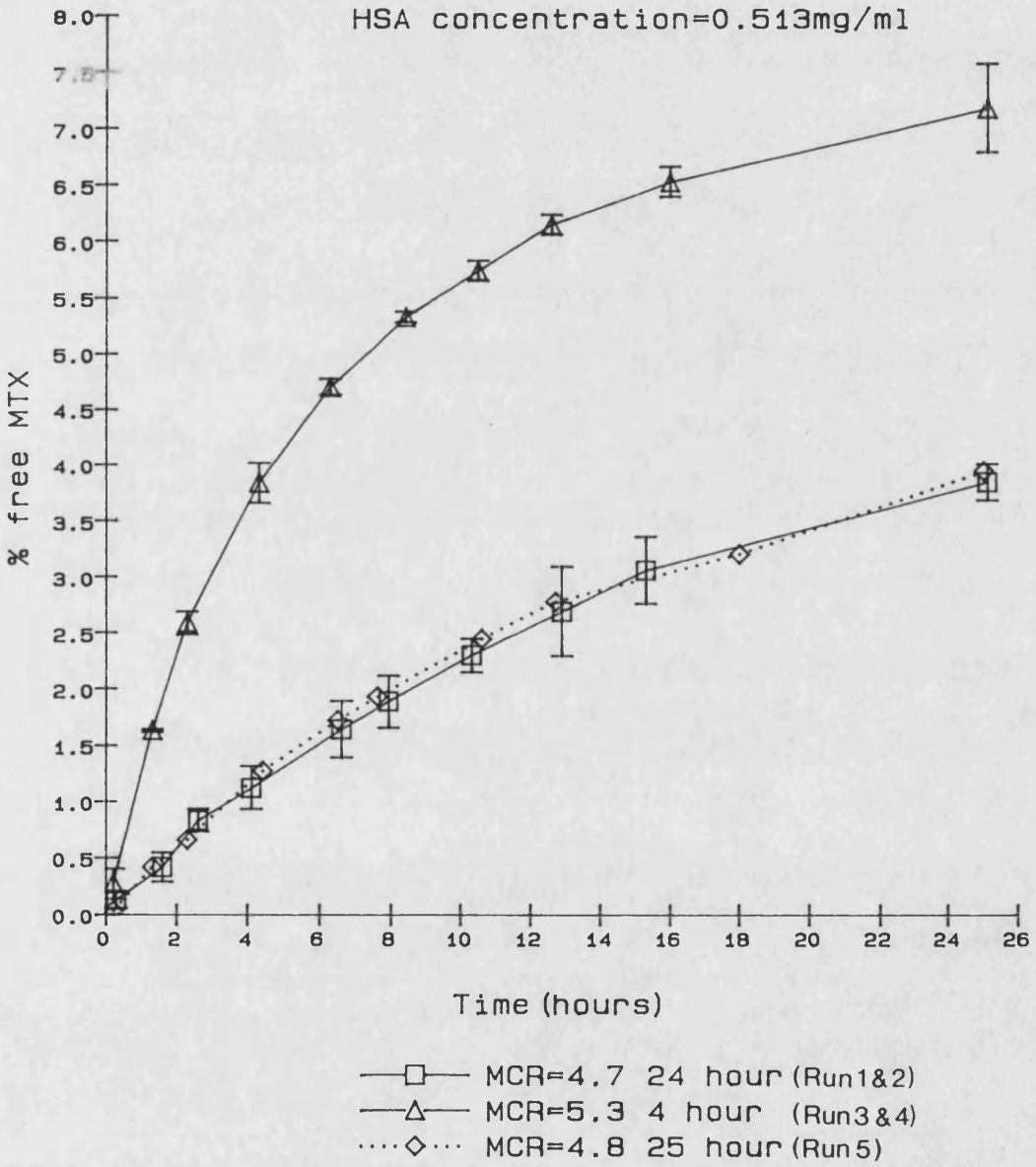
The results suggested that a shorter reaction time was associated with a faster release of free MTX from the conjugate following fractionation (Table A8.2.9 and Figure 2.17). This may be explained with reference to the experiment described previously relating MCR to reaction time which showed that the conjugation reaction was 80% complete at 4 hours. Further storage time could have resulted in cleavage of less stable bonds which would not have been replaced as the ECDI reagent was exhausted. The MTX cleaved after extended reaction time at 25°C would have been removed by fractionation on the Sephadex column resulting in less release of free drug upon subsequent storage at 37°C.

2.4.3 The effect of refractionating a degraded MTX-HSA conjugate

The purpose of this experiment was to investigate whether a conjugate that had been held at 37°C for a substantial period of time could be refractionated to yield a more stable sample.

A conjugate was produced as previously detailed, fractionated on a

Fig.2.17 Release of free MTX from MTX-HSA conjugates with different conjugation reaction times (37°C, PBS pH7.4)



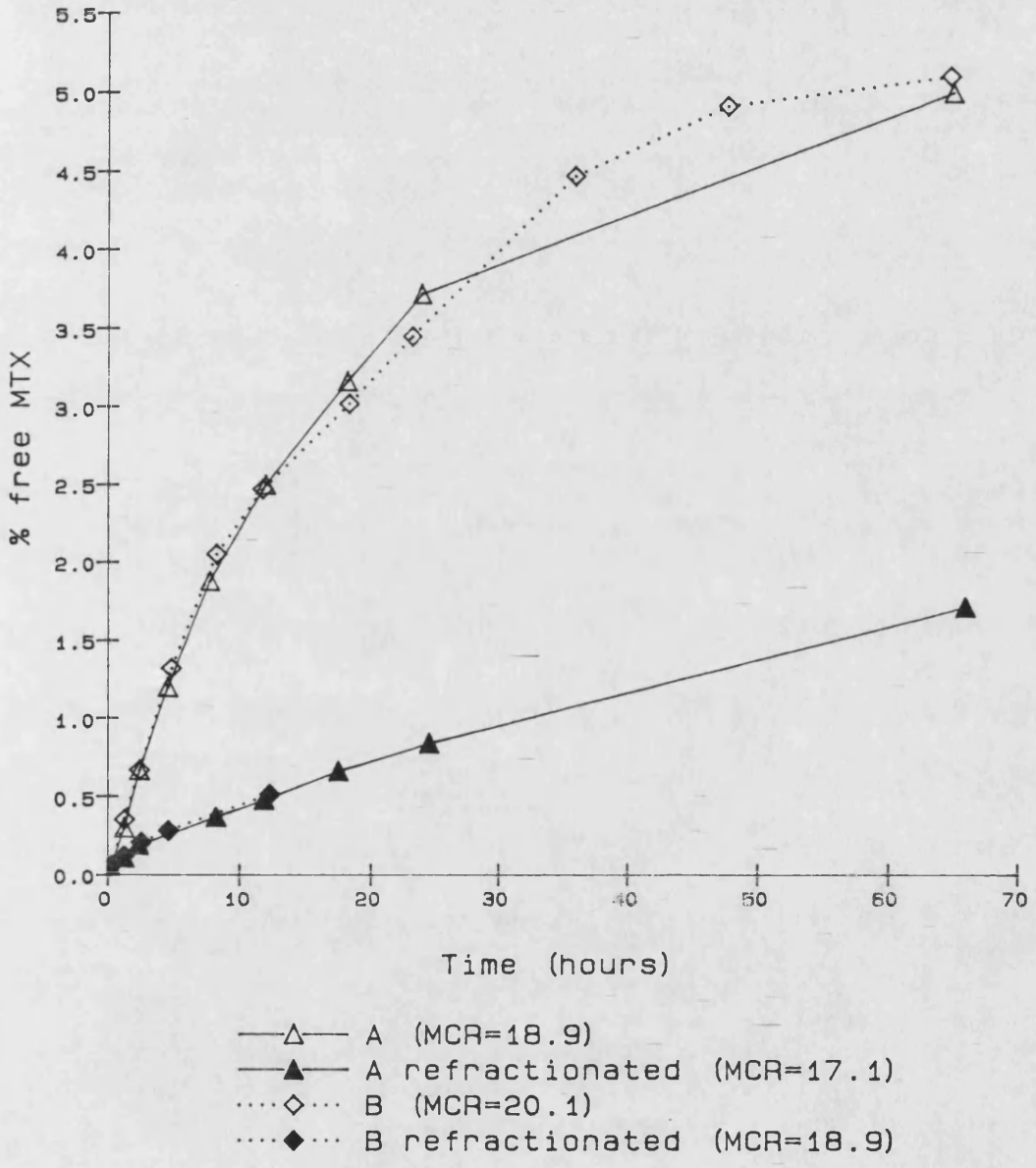
Sephadex column, stored at 37°C and then repeatedly injected on to a Superose 12 column. The sample was held at 37°C for approximately 65 hours and was then refractionated on the Sephadex column (5.13ml injected, 100ml collected). Its stability was reassessed using the Superose 12 system. Detection in both cases being either at 300 or 370nm. Samples '1' and '2' represented the freshly fractionated sample and the refractionated samples respectively. The experiment was repeated on a conjugate with a similar MCR. The second fractionation was performed following storage at 37°C for 60 hours on a short Sephadex column (5.13ml injected, 20ml collected). Data is shown in Table A8.2.10 and Figure 2.18.

The MTX-HSA peak was found to be smaller following the second fractionation due to the dilution effect but its shape was essentially unaltered. It was demonstrated that refractionation of a degraded sample drastically reduced the rate of release of free MTX but did not prevent it completely, implying that MTX-HSA bonds remained which were hydrolysed at a slower rate.

2.4.4 The effect of pH on the release of MTX

The purpose of this experiment was to determine whether the pH of the buffer affected the rate of release of MTX from the conjugate. Conjugate solutions were prepared by fractionating a 1ml sample of reaction mixture (allowed to react at 25°C for 24 hours and then frozen at -18°C until required) using distilled water as the eluant. Aliquots of the conjugate fraction (MCR = 9.92) were then mixed with an equivalent volume of double

Fig.2.18 Effect of refractionation on the release of free MTX from MTX-HSA conjugates. (37°C, PBS pH7.4)



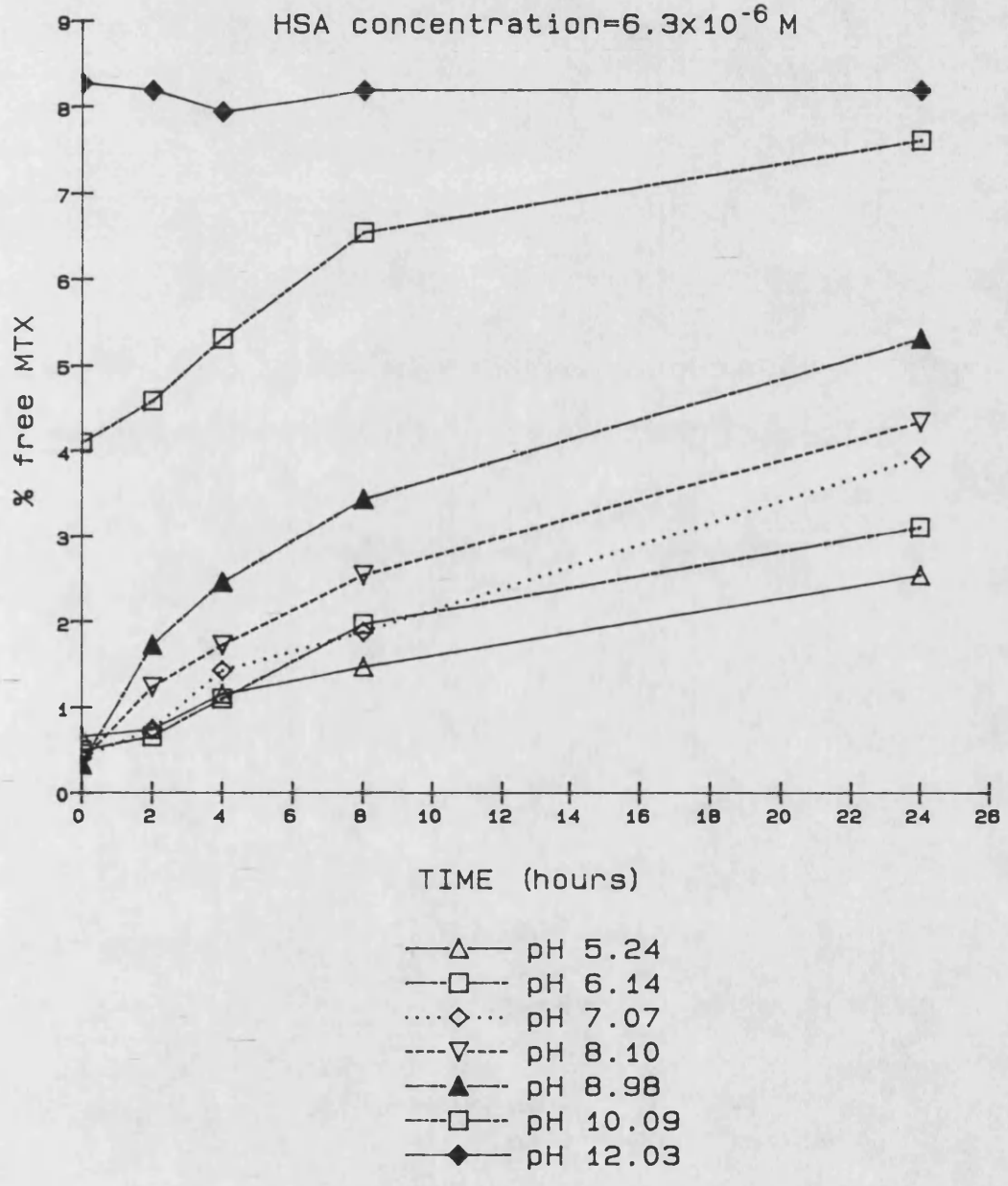
strength buffer, incubated at 37°C for the required period of time and then frozen prior to assay on the TSK column. Data are listed in Table A8.2.11 and plotted in Figure 2.19. A pH 4 sample was also produced, however this pH caused precipitation of MTX-HSA from solution and the sample was not subsequently assayed.

The buffer solutions alone were chromatographed with the result that pH 5, 6 and 7 produced a deflection at 300nm but only at a low level. The retention time of the solvent was found to be between that of the conjugate and free MTX and consequently would not have interfered with the assay. The peak heights, shape and area were similar at all pH conditions except at pH12 which produced a much broader peak for MTX-HSA with a slightly reduced area (mainly accounted for by the large release of MTX). The change in peak shape at pH 12 was thought to be due to an alteration in the tertiary structure of HSA.

The results demonstrated that an increase in pH brought about a more rapid and extensive release of MTX up to a limit of about 8% at pH12. The rapid plateauing at pH12 inferred that 8% was the maximum amount released after the conjugation had been allowed to proceed for 24 hours at 25°C. The samples stored at other pH values tended towards this limit after longer incubation at 37°C (Figure 2.19). This implied that the hydrolytic degradation of MTX-HSA conjugates was base-catalysed.

In an attempt to synthesise a stable product (using ECDI) the conjugation reaction (MCR = 10) was performed in water, stored at 4°C for at least 24 hours, then the pH raised to 11. The solution was stored at pH 11 and 4°C for up to 23 hours prior to fractionation in PBS at pH 7.4. The samples

Fig.2.19 Effect of pH on the release of free MTX from MTX-HSA conjugates (37°C)



were then incubated at 37°C and the release of free MTX analysed by HPLC (Table 2.8). The increase in pH had no effect on the shape of the chromatogram.

Table 2.8 Release of free MTX from conjugates following storage at pH 11 prior to fractionation

Incubation time at pH 7.4, 37°C	% release of free MTX Storage time at pH 11, 4°C (hours)						
	0	1	2	3	4	6	23
0	5.2	3.7	1.7	0.0	0.0	0.0	0.0
2	6.2	3.8	1.9	0.2	0.2	0.2	0.3
4	6.9	3.8	1.9	0.0	0.3	0.3	0.3
24	9.8	6.8	5.4	0.5	0.7	0.6	0.7

It can be seen from Table 2.8 that incubation at pH 11 for 3 hours and more drastically reduced the release of free MTX to approximately 0.5% over 24 hours at pH 7.4 and 37°C.

2.4.5 Stability of MTX-HSA conjugates produced using the active ester

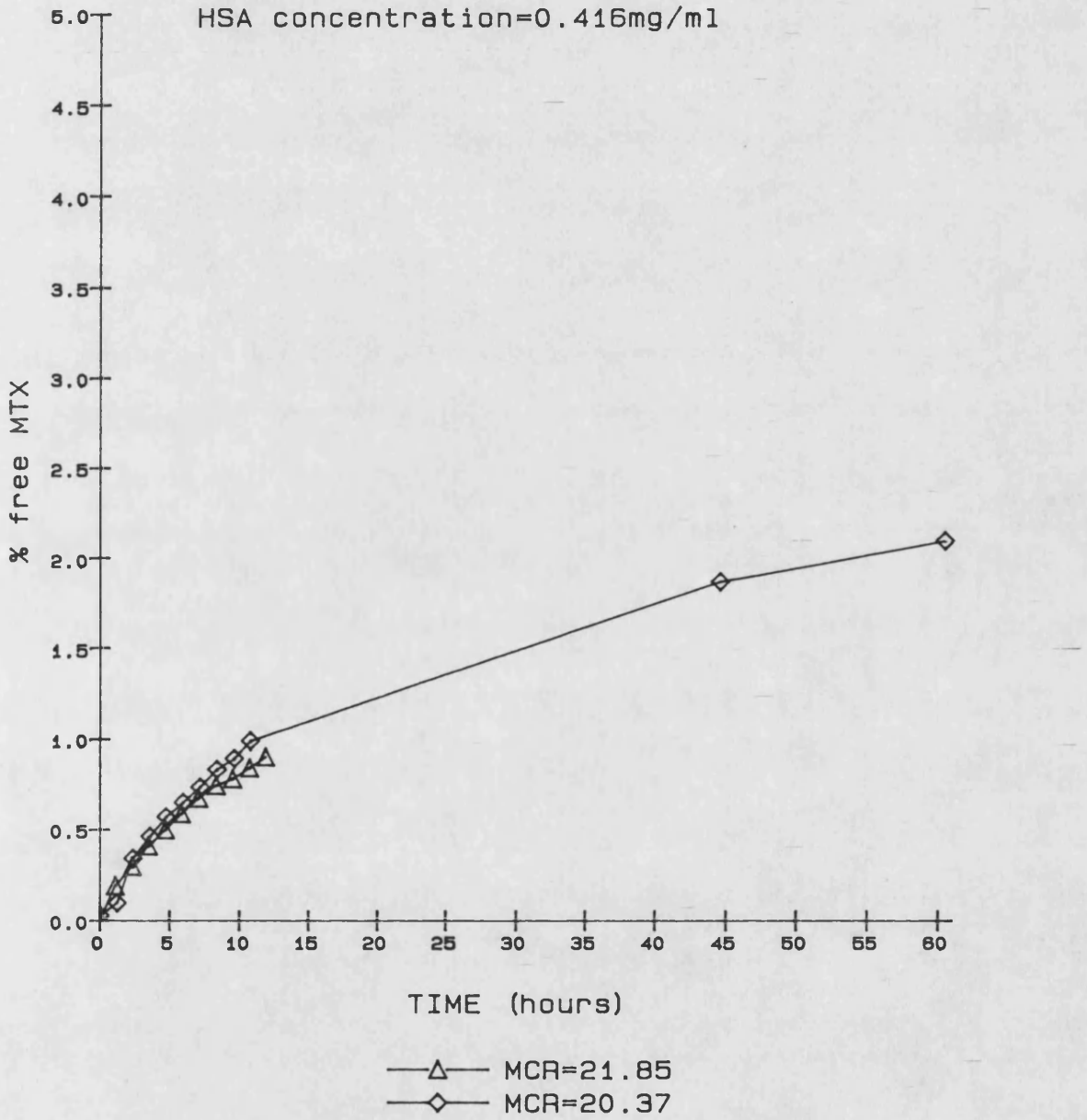
MTX-HSA conjugates were prepared using the active ester derivative of MTX and separated as previously described. The stability of these conjugates in buffer (PBS, pH 7.4) at 37°C was assessed using Superose 12 column. Data is shown in Table A8.2.12 and plotted in Figure 2.20.

2.4.6 Discussion

Initial experiments with MTX-HSA conjugates, where the reaction mixture was separated on the GPC column and then dialysed against buffer at ambient temperature and lyophilised, showed upon FPLC analysis that there was a significant level of free MTX present in the reconstituted sample. Further investigations revealed that the conjugate solution immediately post column contained no free MTX; additional storage at elevated temperature revealed the instability of the conjugate. It was decided to use the FPLC systems to quantify the release of MTX from the conjugate at 37°C (dialysis would remove any free MTX and also variable amounts of hydrolysed MTX depending on temperature and time).

The samples were collected from the Sephadex column and further processed as quickly as possible in order to minimise the time between the start of collection and application to the FPLC column. This finite amount of time (15-30 minutes) resulted in a small amount of free MTX being present in the initial sample, these time points are thus quoted as 0.3 hours.

Fig.2.20 Release of free MTX from ester conjugated MTX-HSA conjugates (37 °C, PBS pH7.4)



A comparison of high (MCR = 20) and low (MCR = 5) strength conjugates at pH7.4 and 37°C reveals that a similar level of free MTX (expressed as a percentage of total MTX) was released from both conjugates. The release of MTX (3.5 - 4.0%) was not dependant upon the MCR of the conjugate. It may be noted from the shape of the curves that there is an initial fast rate of release which starts to tail off after about four hours to result in a slower residual rate of release after about 16 hours (Figure 2.16). This would imply that there are two populations of hydrolysable bonds formed between MTX and HSA, one group which degrades faster than the other giving rise to the initial faster rate of release. It would appear that the more labile bonds tend to form as a fixed proportion of the total MCR (overall percent release is similar) and are dependent on the relative levels of ECDI and MTX. The chromatograms show that the conjugate peak maintains its characteristic shape during the degradation experiments and release was not due to break down of the molecule or deterioration of the tertiary structure (see Figure 3.6).

The results from the shorter reaction mixture incubation time experiment (4 hours) show a higher level of release (7%) than those reaction mixtures left for 24 hours (Figure 2.17). The fact that the ECDI solution was not freshly prepared for runs 3,4, and 5 resulted in reduced MCR's, as expected, but this has had no effect on the release of free MTX. The 25 hour sample had the same release profile as other conjugates produced with freshly prepared ECDI solutions and incubated for a similar period. The increase in release observed with the 4 hour reaction time sample may in part be explained by the decreased reactivity of the carbodiimide with storage time in aqueous solution. In the reaction mixture the labile bonds will be formed and then slowly hydrolysed, as the carbodiimide

becomes exhausted these links will not be reformed (since there is excess MTX). Consequently at longer incubation times more of the labile bonds will have been hydrolysed and a lower rate of release will be detected from the isolated conjugate. It can be seen from this that the storage time and temperature will have a profound effect on the stability of the conjugate post-fractionation.

The samples which had been fractionated then stored at 37°C for 65 hours prior to refractionation on a Sephadex column showed a much slower rate of release than samples from the initial fractionation (Figure 2.18). The rate of release of drug was faster than in the later stages of the first part of the experiment. This may be due to a disturbance in the equilibrium or possibly a dilution effect, however similar release rates were observed for refractionations A and B where B was 5 times the concentration of A. It would suggest that the bonds being broken are the less labile ones.

The effect of altering the pH of the buffer brought about changes in the release profile. The results demonstrate that an increase in pH brings about a more rapid and extensive release of MTX up to a limit of about 8% at pH12. This implies that the hydrolytic degradation of the more labile bonds of MTX-HSA conjugates was base catalysed. Acid catalysis could not be examined due to insolubility. Incubation of the reaction mixture at pH 11 prior to fractionation (pH 7.4) reduced the level of release of the resulting conjugate solution to about 0.5% (free MTX after 24 hours at 37°C. This was similar to that observed for refractionated samples and conjugates produced by the active ester method.

Release experiments from active ester mediated conjugates revealed a much slower release rate after a reaction time of only 5.5 hours indicating a more stable product (Figure 2.20). The release profile was similar to those observed following refractionation of the carbodiimide-mediated conjugate (Figure 2.18) indicating a lack of the more labile bonds.

CHAPTER THREE: ENZYMATIC DEGRADATION OF MTX-HSA CONJUGATES

3.1 Introduction

The release of drugs from macromolecular carrier systems in the presence of enzymes has previously been examined however little attention has been focused on the integrity of the carrier itself following exposure to enzymes (Trouet et al, 1982).

In this study it was decided to analyse the release of MTX from the carrier system and also the molecular weight distribution of the carrier itself following incubation with trypsin and tritosomal enzymes. Trypsin was examined as a model enzyme since from its known specificity (Keil, 1971) it should have been capable of breaking down the carrier systems it was proposed to use ie HSA and glutamic acid/lysine copolymer. It was thought that this would provide useful information as to whether the attachment of MTX prevented the degradation of the carrier peptide and if the breakdown of the conjugate resulted in an increase in the release of MTX as the free drug.

The question as to whether the conjugate would be degraded by lysosomal enzymes were it to be a lysosomotropic agent was addressed by incubation with tritosomes.

The tritosomes used in this series of experiments were isolated from rat liver according to the method of Trouet et al (1974), these comprise a plethora of enzymes with pH optima varying between 2 and 8 (Barrett, 1972). The lysosomes themselves contain a number of enzymes including nucleases, glycosidases, aryl sulphatases, lipases, phospholipases, phosphatases and and proteases. The latter are capable of dismantling

proteins and polypeptides; they comprise the cathepsins and also acrosin which is an endopeptidase with a specificity similar to trypsin. It was hoped to discover using FPLC whether MTX was released as free drug or linked to an oligopeptide unit following tritosomal degradation. It has previously been suggested that spacer units may be necessary to enable enzymatic hydrolysis and release of the free drug (Trouet et al, 1982); this may be required for drugs such as DNR but MTX has been shown to be active in the polyglutamated form and even when bound to a polypeptide.

Trypsin

Trypsin is a serine protease which preferentially catalyses the hydrolysis of ester and peptide bonds involving lysine and arginine (basic amino acids). The inactive precursor, trypsinogen, is synthesized and subsequently converted to trypsin in pancreatic cells. Trypsinogen is converted to the active enzyme by limited proteolysis at pH8 (catalysed by small amounts of trypsin, enterokinase or thrombin), autocatalysis also occurs under mildly alkaline conditions.

The molecular weight of the enzyme has been calculated as 23,985 (from known structure of bovine trypsinogen) and 23,560-23,700 by physicochemical measurements; the extinction coefficient E1%/1cm is 15.4 at 280nm.

The solution stability was determined by Wang and Carpenter (1967) who showed that after 2 hours at pH 8 trypsin retained 100% of its original activity (on Nα-benzoyl-L-arginine ethyl ester as substrate) and 92% after 20 hours.

The catalytic site itself is dependent on particular serine and histidine residues whereas it is assumed that negatively charged residues are present in the specificity site enabling electrostatic binding of positively charged substrate groups. As previously mentioned the trypsin specifically cleaves bonds involving the carboxyl group of lysine or arginine with a pH optimum lying between pH7 and 9. It is suggested that the enzyme exhibits increased activity towards the arginine residues but this may be attributed to the adjacent residues (Wang and Carpenter, 1967). The rate of catalysis may be reduced by removal of the positive charge on the lysine either by altering the pH (pKa of the guadinium group on arginine is much higher) or by a substituent on the ϵ-amino group. Other factors which may affect the hydrolysis are the tertiary structure of the substrate (although positively charged groups are normally located on the surface of native proteins) and the presence of negatively charged groups in the vicinity of the labile bond.

3.2 The effect of trypsin on the release of MTX from MTX-HSA conjugates

In this experiment the conjugation reaction was set up as previously described (section 2.2.5) using a (1:118:173 ratio, pH 7.4) and then fractionated on a Sephadex column (mobile phase PBS, pH 7.4) after 24 hours at 25°C. A 50ml aliquot of the conjugate solution was taken and 1ml of a trypsin solution (c. 2.5mg/ml) was added to it prior to incubation at 37°C. The samples were then analysed on a calibrated Superose 12 column at time intervals up to 24 hours.

The conjugate peak was found to be degraded into a series of lower molecular weight peaks as well as free MTX which eluted separately from the other peptide peaks, the retention time of which was identified from MTX calibration samples (Figure 3.3). Data are shown in Table A8.3.1 and plotted in Figure 3.1. The experiment was performed at least in duplicate.

The results from this experiment demonstrated that the release of MTX from the conjugates was not greatly affected by incubation with trypsin although the rate appeared to be increased slightly by the addition of enzyme. The extent of the release was not affected by the enzyme since following 45 hour incubation at 37°C the same conjugate solution was found to contain 5.4% free MTX both in the presence and absence of enzyme.

A further experiment was performed by exposing a conjugate solution to different levels of trypsin and monitoring the release of MTX. The conjugate (MCR = 22.1) was produced as normal and following fractionation in PBS pH 7.4 had the following added to 25ml aliquots:

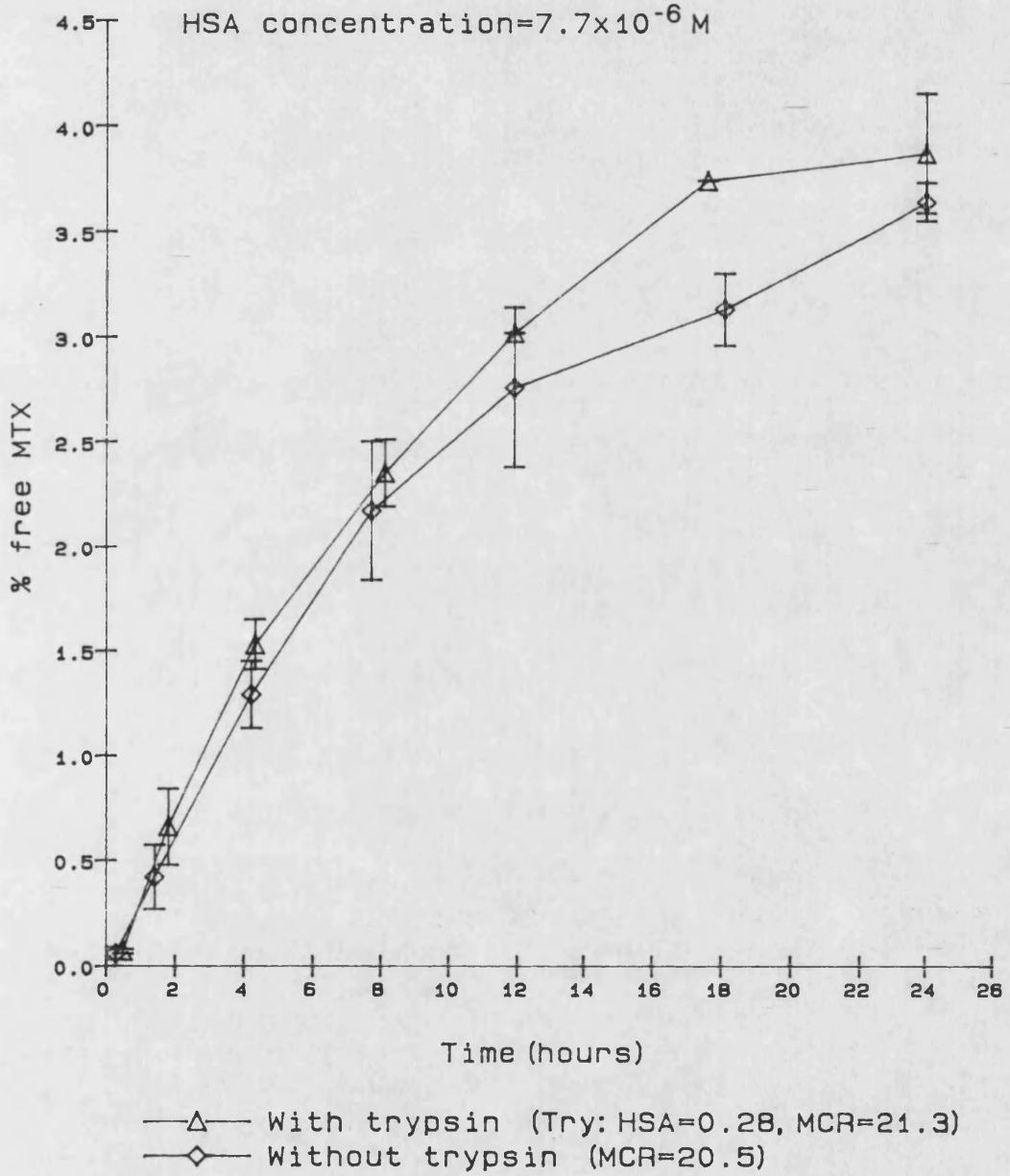
A - 13.0mg trypsin, HSA conc. = 7.752×10^{-6}M,
Try:HSA molar ratio = 2.82:1

B - 1ml of a 13.5mg/ml solution of trypsin in PBS pH 7.4 giving HSA conc. = 7.454×10^{-6}M, molar ratio = 0.29:1

C - 0.1ml of 13.5g/ml solution of trypsin in PBS pH 7.4 giving HSA conc = 7.721×10^{-6}M, molar ratio = 0.028:1

NT - conjugate sample with no trypsin added, HSA conc = 7.752×10^{-6}M

Fig.3.1 Effect of trypsin on the release of free MTX from MTX-HSA conjugates (37°C, PBS pH7.4)



Subsamples were placed in sterile vials, sealed and incubated at 37°C prior to assay on the Superose column at 300nm. The release of MTX was calculated from the peak heights compared to standard samples.

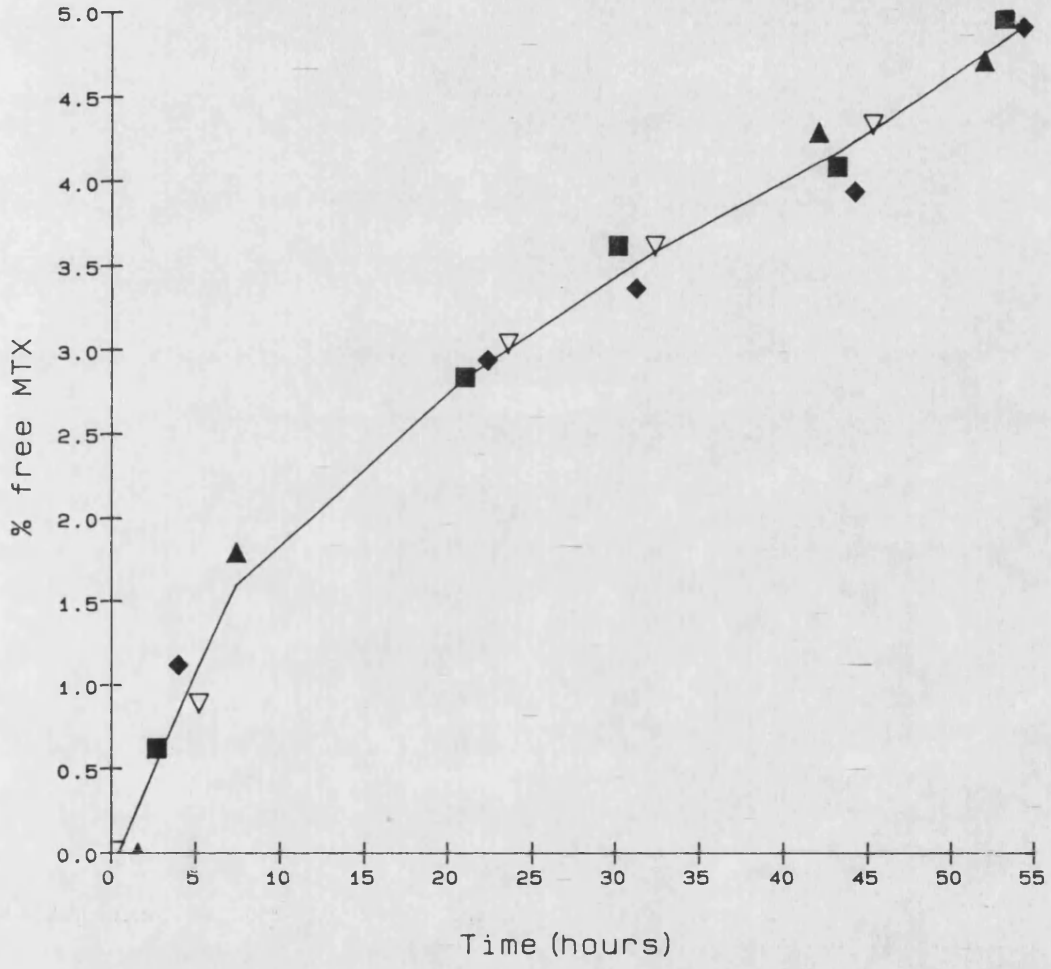
The data (Table A8.3.2, Figure 3.2) confirmed the previous result that the addition of trypsin had little effect on the release of MTX from the conjugates in that all the points lay on virtually the same curve. The addition of further enzyme at the end of the run had no effect on the release of MTX from either A,B or C.

The release of MTX in this case was slightly less than previously observed possibly due to the reaction conditions which were 24 hours at 25°C followed by storage at -18°C for 24 hours prior to thawing and fractionation.

3.3 The effect of trypsin on the molecular weight distribution of HSA

In order to investigate the digestion of HSA by trypsin, suitable enzyme solutions were produced in PBS pH 7.4 and added to an HSA solution (PBS pH 7.4) in order to produce molar ratios of HSA/trypsin of 1/2.81 (high level) and 1/0.28 (low level). The final HSA concentration was 0.45mg/ml. Blank solutions were also prepared of HSA and trypsin alone in PBS pH7.4. The samples were incubated at 37°C for 20 hours and then frozen along with the initial samples prior to assay using a TSK column with detection by UV at 279nm. The chromatograms were analysed for molecular weight distribution and peak area using the BBC microcomputer utilizing the

Fig.3.2 Effect of different levels of trypsin on the release of free MTX from MTX-HSA conjugates ($37^{o}C$, PBS pH7.4)



▲ Sample A (Try: HSA=2.82) HSA=7.75×10^{-6} M
■ Sample B (Try: HSA=0.29) HSA=7.45×10^{-6} M
◆ Sample C (Try: HSA=0.03) HSA=7.72×10^{-6} M
▽ NT (no trypsin) HSA=7.75×10^{-6} M

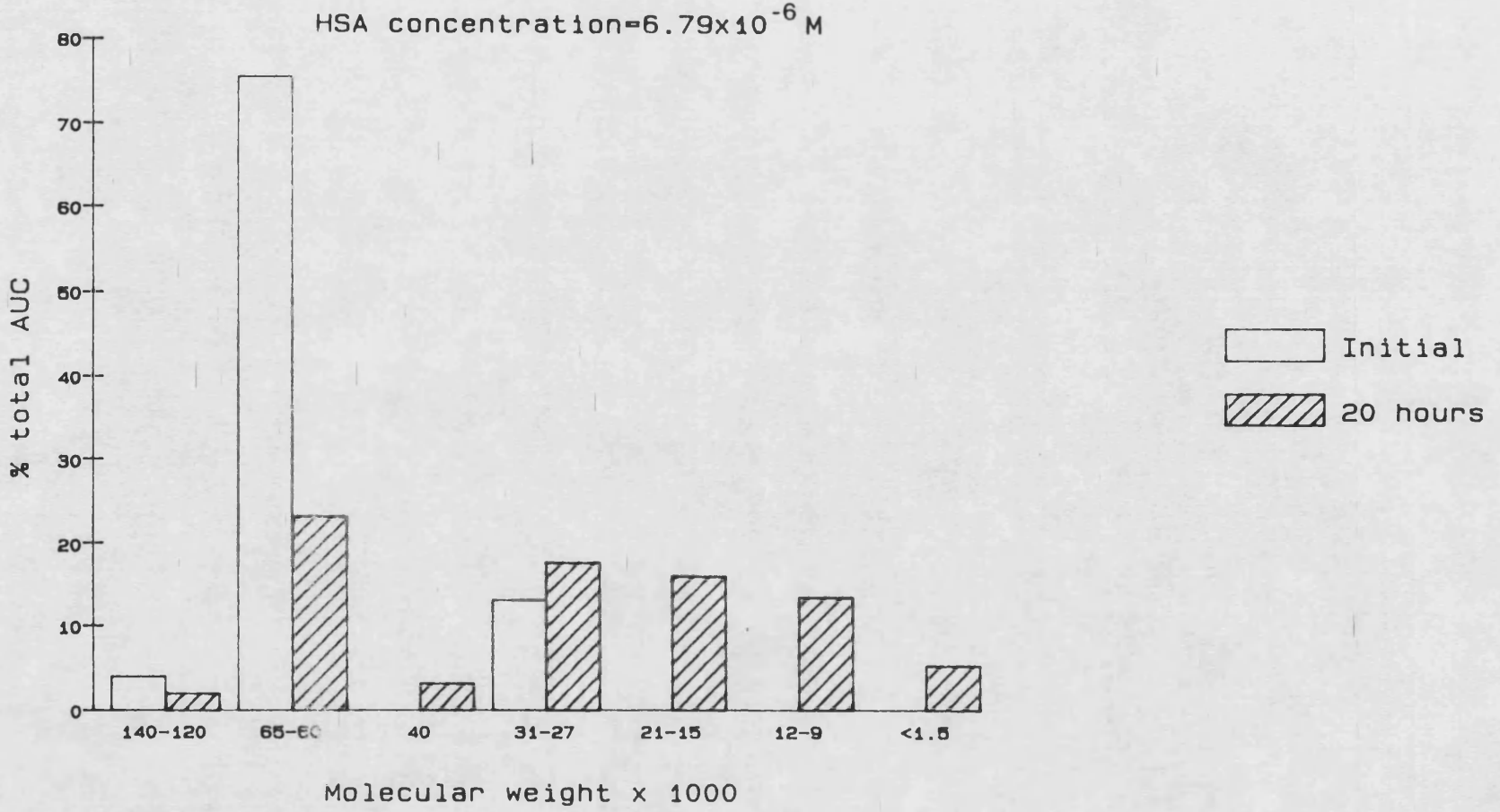
chromatogram subtraction program. This was necessary since the trypsin chromatogram interfered with that for HSA, the effect being quite marked at the elevated level of trypsin. The blanks employed for subtraction were the relevant level of trypsin both initially and following 20 hour incubation. The efficiency of the subtraction procedure can be demonstrated by the reduction in total area (since the trypsin especially at the higher level had a significant effect on the area under the curve).

Trypsin concn.	AUC as % of HSA control	
	before subtraction	after subtraction
high level	341	103
low level	122	100

Analysis of the trypsin samples revealed that autodigestion had occurred after 20 hours, the effect being far more pronounced at the higher concentration of enzyme.

The results of the HSA degradation experiment, displayed in Table A8.3.3 and Figure 3.3, showed that trypsin was capable of dismantling the protein molecule into a series of discrete peaks of lower molecular weight. The higher level of enzyme predictably resulted in a larger percentage of lower molecular weight material during the fixed reaction time. It is interesting to note that about 5% of the material was retained by the column with a Kav > 1 indicating that some degree of interaction was occurring between the peptidic fragments and the column support material.

Fig.3.3 Effect of trypsin on the molecular weight distribution of HSA (37°C, PBS pH7.4, 20 hours) (Try: HSA=2.82: 1)



The HSA control samples exhibited little alteration in the shape of the chromatogram upon storage with the monomer peak remaining at around 85% of the total AUC. It may be noted that levels of the monomer are lower than this for the initial analyses of low and high level trypsin which is proposed to be due to the finite amount of time between the addition of the trypsin solution and the injection time (or time of freezing) resulting in partial degradation (lower dimer and monomer peaks).

3.4 The effect of trypsin on molecular weight distribution of MTX-HSA

The samples analysed in this experiment were those analysed in section 3.2 which are summarised below:

MCR = 22.1 in PBS pH 7.4

Molar ratio Try/HSA A: 2.82/1

B: 0.29/1

C: 0.028/1

NT: 0/1

The signal from one of the channels was logged onto the microcomputer to enable subsequent analysis of the molecular weight distribution employing calibration values generated throughout the analysis period.

The chromatograms were analysed without subtracting the trypsin chromatogram since the absorbance due to the enzyme at 300nm was very low and would not have had a significant effect on the output from the

Table 3.1: The effect of varying levels of trypsin on the molecular weight distribution of MTX-HSA conjugates at pH 7.4 (37°C)

Values expressed as a percentage of the total area under the curve

Time	Sample A (2.82)*					Time	Sample B (0.29)*					Time	Sample C (0.03)*					Time	NT				
	Peak No						Peak No						Peak no						Peak No				
(Hours)	1	2	3	4	5	(Hours)	1	2	3	4	5	(Hours)	1	2	3	4	5	(Hours)	1	2	3	4	5
1.6	5.5	49.5	27.2	10.5	3.7	2.7	8.9	60.4	17.8	8.1	3.3	4.0	12.1	69.1	10.8	5.3	1.5	5.2	21.8	75.8	-	-	-
7.4	2.0	21.6	32.8	17.7	13.0	8.5	6.0	51.9	24.1	10.5	5.1	9.8	11.9	65.7	12.2	6.5	2.5	10.8	22.6	74.6	-	-	-
29.0	0.5	10.9	33.5	20.8	20.8	30.1	3.1	29.6	28.8	14.4	11.5	31.2	8.0	54.7	20.0	8.6	6.5	32.3	19.7	72.0	-	-	-
42.0	0.2	8.8	32.2	22.3	23.4	43.1	1.9	24.5	30.6	15.7	13.2	44.2	6.1	45.0	25.5	9.8	8.1	45.2	19.1	73.2	-	-	-
-	-	-	-	-	-	56.5	1.9	20.0	30.8	17.0	16.4	54.2	5.6	39.4	21.8	11.8	10.3	-	-	-	-	-	-

Peak No 1 - HSA dimer MW = 140-160,000
2 - HSA monomer MW = 60-70,000 (initially)
3 - MW = 15-20,000
4 - MW = 4,000
5 - MW = <1,500

* = molar ratio of trypsin to HSA
NT = No trypsin

HSA Concentration Sample A = 7.75×10^{-6}M
Sample B = 7.45×10^{-6}M
Sample C = 7.72×10^{-6}M
NT = 7.75×10^{-6}M

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detector. A trypsin sample (1.35mg/ml) gave a barely detectable peak. The total areas expressed as a % of the total area of control (no trypsin) were as follows:

A - 107%

B - 102%

C - 101%

The samples were incubated at 37°C and were assayed (without further storage) at various time points over a 60 hour period.

The addition of trypsin to the conjugate resulted breakdown of the main MTX-HSA peak to a series of lower molecular weight fragments (up to about 7) clearly distinguishable as separate peaks on the chromatogram. This was not the case for the sample without trypsin in which the only alteration over 45 hours was the expected increase in the level of free MTX. The results are displayed in Table 3.1.

The action of the enzyme brought about a reduction in the molecular weight of the carrier protein. The high molecular weight dimer and monomer being broken down into peptides approximately equivalent to 15,000 and 4,000 with a significant amount being retained after B12 and having a Kav in excess of 1. This suggests that low molecular weight material (less than c.1,500) was being adsorbed by the column in a similar manner to free MTX. The extent of degradation to below MW 1,500 can be illustrated from sample A which after 42 hours was composed of 23% peptidic MTX in this fraction with only 9% remaining as monomer.

The addition of further trypsin and incubation for an extra 30 hours resulted in more extensive degradation, 43% of which was below MW 1,500. It was found that trypsin digested the protein into lower molecular weight fragments whose molecular weight also diminished with incubation time, for example the monomer peak changed from 67,000 to 54,000 after 42 hours (Sample A).

The degradation pattern was similar at all levels of trypsin but as expected the rate was greater as the Try:HSA ratio was increased. The peaks from a degraded sample (A) were analysed using the detector in scanning mode which showed peaks in the spectrum corresponding to those of MTX. The fractions (7) were also collected and then analysed using a scanning spectrophotometer. All fractions exhibited a peak at 370nm indicating that all contained MTX at one level or another. An investigation of the absorbance ratio 370/280nm revealed the following:

Table 3.2 UV analysis of trypsin-degraded fragments of MTX-HSA

Fraction	Retention time	Molecular weight	ratio 370/280nm
1	26.0	50,000	0.32
2	32.5	25,000	0.32
3	34.0	7,000	0.18
4	38.0	3,400	0.36
5	39.5	1,700	0.28
6	45.0	1,000	0.36
7	50.0	MTX	0.42
MTX	50.0	-	0.40
MTX-HSA	24.3	-	0.39

The higher ratios ie. approaching 0.4 suggest a high loading of MTX eg the conjugate value of 0.39. Those with a lower ratio have a relatively higher absorbance at 280nm inferring the presence of more peptide than MTX.

A repeat of this experiment was performed by adding HSA and MTX solutions (PBS pH 7.4) to undissolved ECDI. The reaction mixture was left at ambient for 2 hours followed by refrigeration overnight prior to fractionation. This had the effect of producing a conjugate with a high MCR (25), an increased degree of dimerisation and which released a high level of free MTX.

A solution of trypsin (0.5ml of 25.3mg/ml, PBS pH 7.4)) was added to 25ml of the conjugate solution (molar ratio Try:HSA = 2.74) and the sample filtered (0.2um) into sterile vials; the conjugate solution alone was also filtered into sterile vials and both stored at 37°C prior to assay on the Superose 12 column. A trypsin blank was also produced using 0.5ml trypsin solution and 25ml of buffer. The initial and early trypsinised samples (up to 8 hours) were run at the time point, the remainder of the samples were frozen prior to assay along with calibration standards.

The enzymatically degraded samples had the trypsin chromatrogram subtracted from them prior to computer analysis. The mean area under the curve for the samples with trypsin was found to be 59.27 ($\pm$1.88) which was 103% of that for samples without trypsin 56.67 ($\pm$1.33). The results are shown in Table 3.3 and Figure 3.4.

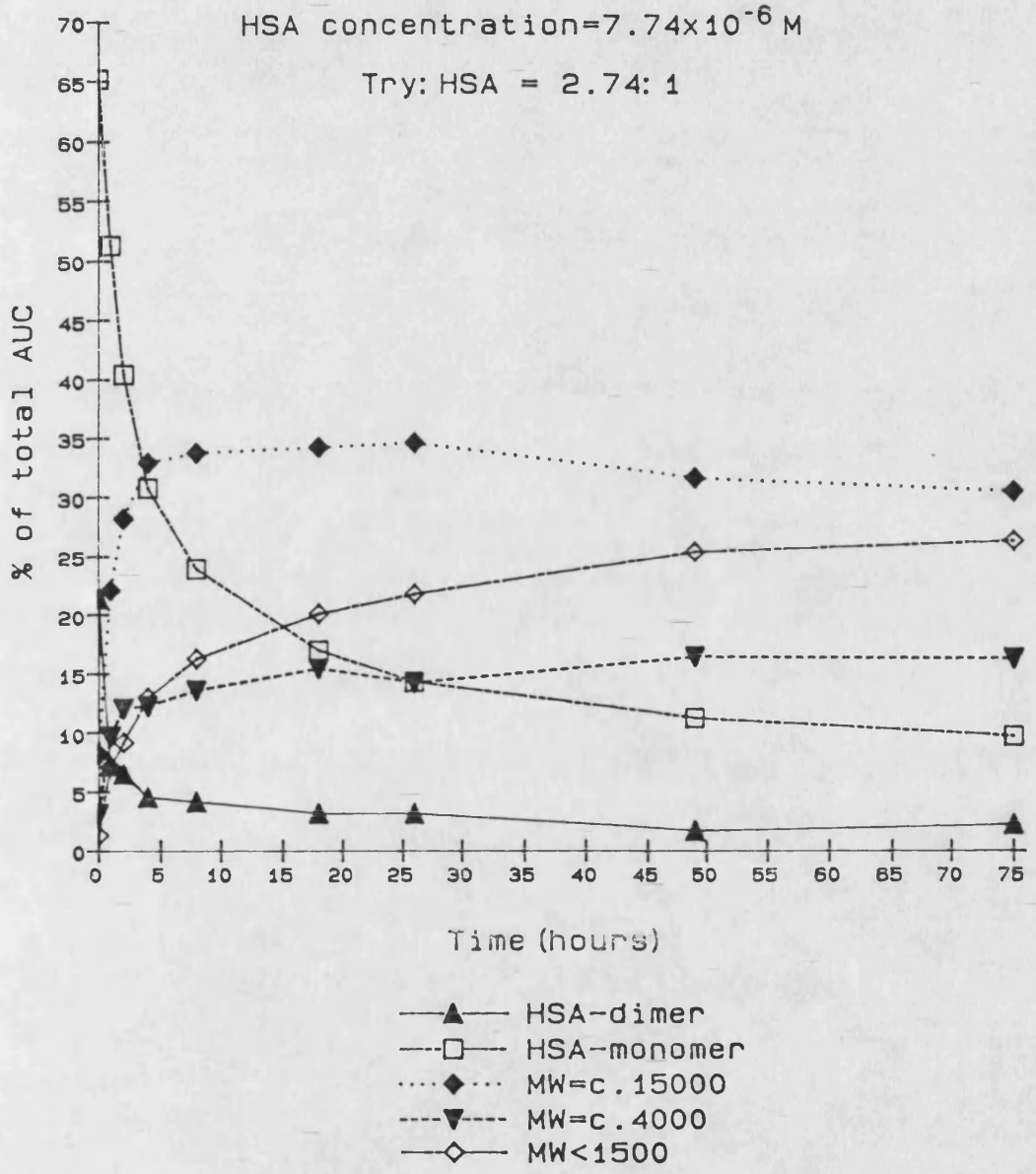
Table 3.3:
The molecular weight distribution of MTX-HSA conjugates in the presence and absence of trypsin in buffer solutions (pH 7.4) at 37°C

	TRYPSIN										NO TRYPSIN			
Peak No	1		2		3		4		5		1		2	
Time (Hours)	%	$MW_x 10^3$	%	$MW_x 10^3$	%	$MW_x 10^3$	%	$MW_x 10^3$	%	$MW_x 10^3$	%	$MW_x 10^3$	%	$MW_x 10^3$
Initial	21.4	215	65.2	67	8.2	20	3.1	3	1.3	<1.5	26.1	190	71.2	69
1	7.2	204	51.3	58	22.1	15	9.7	2.8	7.1	<1.5	26.9	219	66.2	71
2	6.5	182	40.4	56	28.2	15.8	12.1	2.8	9.2	<1.5	27.2	223	66.0	71
4	4.5	190	30.8	56.5	32.9	16	12.3	3	13.0	<1.5	25.6	217	67.9	73
8	4.1	187	23.9	54.5	33.8	15.5	13.6	3	16.3	<1.5	25.0	209	65.7	72
18	3.1	166	17.0	53	34.2	14.5	15.4	3	20.1	<1.5	24.3	213	66.4	72
26	3.1	153	14.3	53.5	34.6	15	14.2	3.8	21.8	<1.5	25.2	219	63.9	72
49	1.7	132	11.2	48.5	31.6	12.3	16.4	3	25.4	<1.5	24.5	215	64.0	72
75	2.2	143	9.7	54.5	30.5	13	16.3	4	26.3	<1.5	24.2	221	63.8	74

results expressed as % of total area

Molar ratio Try:HSA = 2.74
HSA Concentration = 7.74×10^{-6}M

Fig.3.4 Effect of trypsin on the molecular weight distribution of MTX-HSA conjugates ($37^{o}C$, PBS pH7.4)



The release of free MTX (calculated by peak area) was 6.8% after 26 hours, with the release in the presence of trypsin being slightly higher at 8.3% after the same incubation period.

The results from the non-trypsinised sample show that the main MTX-HSA peak which constitutes 65-70% of the total area is relatively unaffected by storage in solution at 37°C. The slight reduction in area being brought about by hydrolysis of MTX with the molecular weight remaining fairly constant at around 72,000. The high molecular weight calculated for the dimer (ie above 140,000) is due to the width of the fraction used to calculate the area under the curve and the molecular weight; it is however at a fairly consistent level (24-27%).

The addition of trypsin to the sample caused a significant reduction in the level of MTX-HSA (dimer and monomer) associated with an increase in material of around 15,000 and 4,000 Daltons. In a similar manner to the previous experiment after 49 hours at 37°C a fairly large proportion of the MTX-peptide fragments were below around MW 1,500 ie. 25% with only 11% remaining as MTX-HSA(monomer).

3.5 The effect of pH on the degradation of MTX-HSA in the presence and absence of trypsin

The conjugate reaction mixtures were prepared as normal and then fractionated using either PBS pH7.4 or McIlvaines buffer pH5.6 as the mobile phase. The degradation experiments were performed in duplicate, the reaction mixtures being stored at -18°C prior to fractionation in the appropriate buffer system; the mean MCR's were found to be 9.3 (±0.2) at pH 7.4 and 9.9 (±0.3) at pH5.6. Aliquots of the fractionated samples were taken and volumes of either buffer or trypsin solutions were added to yield a final concentration of HSA of 1mg/1ml (4.19×10^{-6}M). The molar ratio of trypsin to HSA was 0.73:1.

Samples of these solutions were either frozen immediately after preparation or incubated at 37°C for 24 hours and then stored at -18°C prior to analysis using the TSK column. The samples were analysed in duplicate and calibration standards were used to determine the molecular weight distributions and MTX release; analysis of blank samples enabled chromatrogram subtraction. The results are displayed in Table A8.3.4 and Figure 3.5. An example of the chromatograms from an experiment at pH 7.4 is shown in Figure 3.6.

The MTX-HSA which eluted at the expected retention time (ie c.13.4 mins) was determined as one peak (the dimer not separating as well as on the Superose column) resulting in the initial values approaching 100%. The molecular weight of 59,000 was due to the width of the cut used to determine the area/molecular weight. It has been shown previously that a reduction in the pH results in less MTX release from the carrier system;

Fig.3.5 Effect of trypsin on the molecular weight distribution of MTX-HSA conjugates following incubation at different pH (37°C, 24 hours) (Try: HSA=0.73: 1)

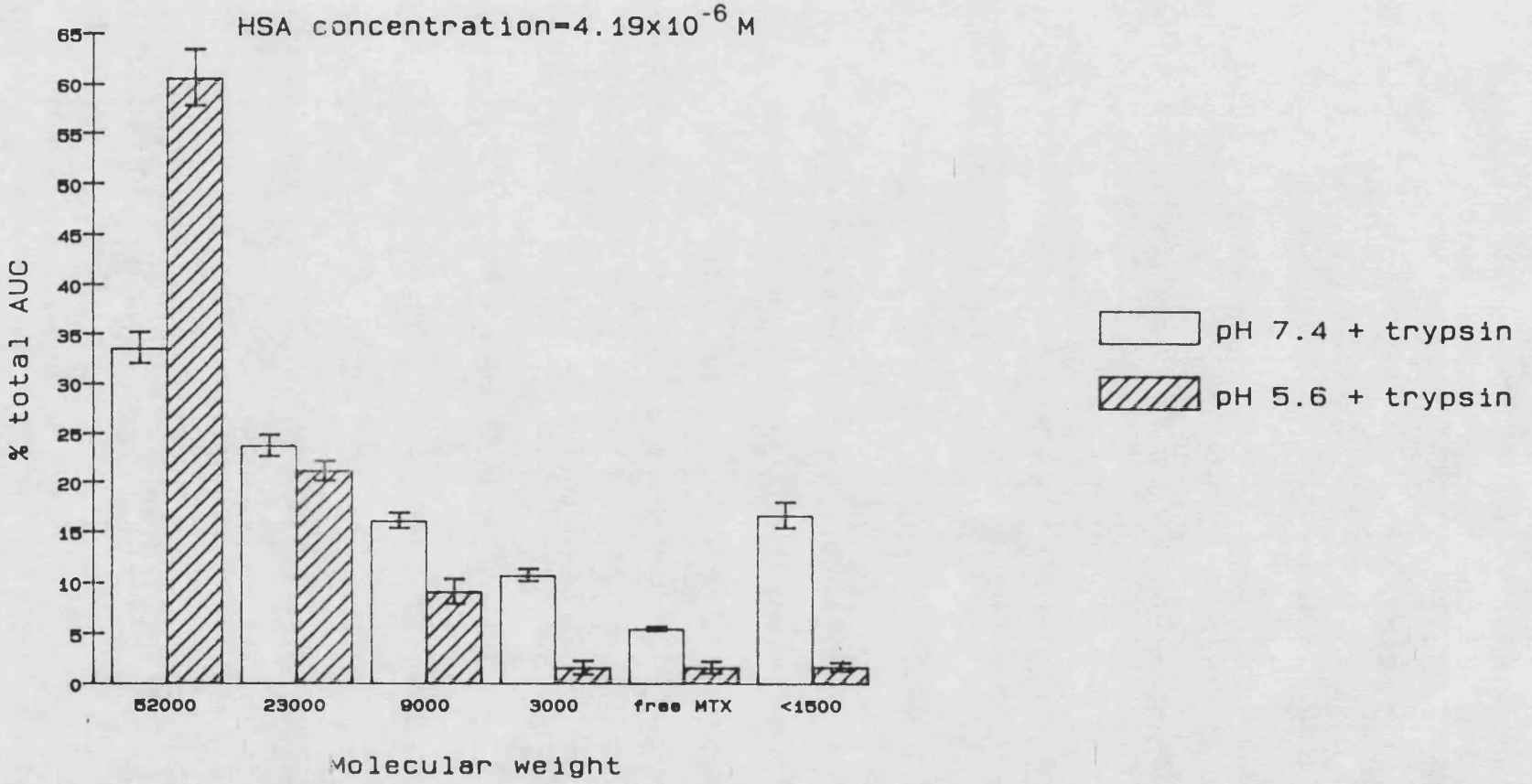
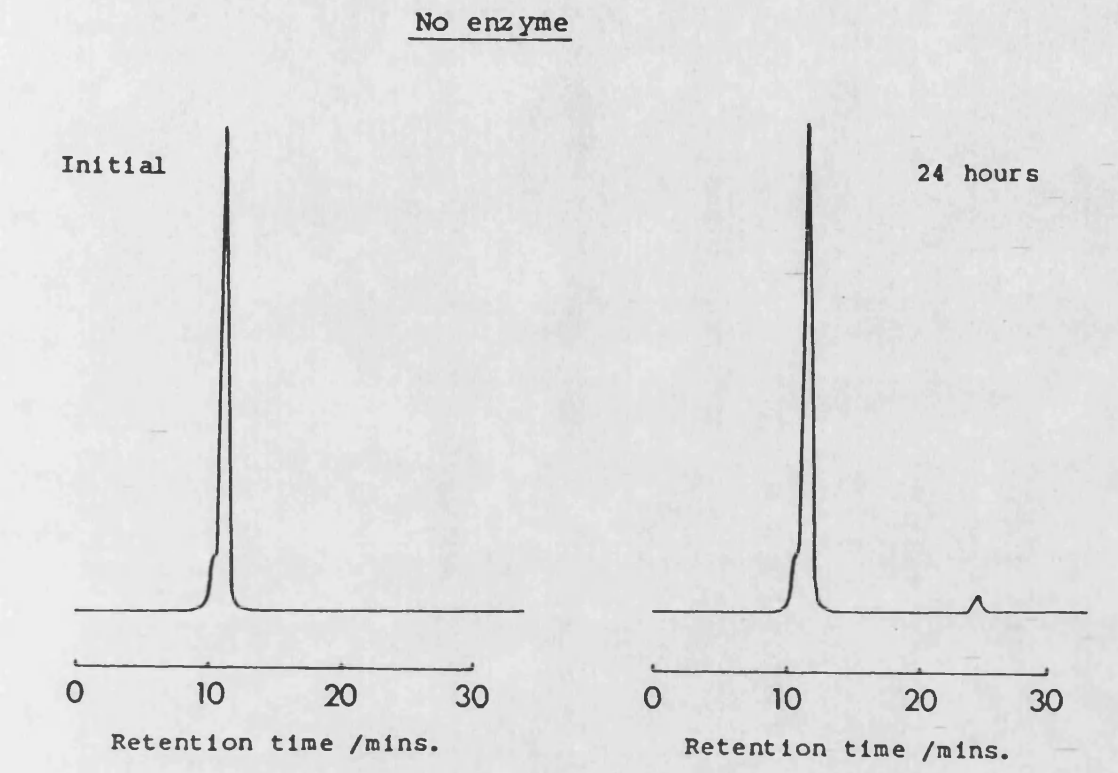


Fig 3.6

In vitro degradation of MTX_{10}-HSA conjugates at pH 7.4 and 37°C



this was confirmed in this experiment with the level of free MTX at pH5.6 being about a third that released at the higher pH.

The addition of trypsin brought about the expected change in the molecular weight distribution with a reduction in the level of intact HSA-MTX and the appearance of low molecular weight MTX-oligopeptides. The lower pH environment resulted in a less extensive dismantling of the conjugate with very little material below about MW 1,500 ie 1.7% as opposed to 16.7% at the higher pH. The digestion of the conjugates at both pH conditions resulted in fragments of a similar molecular weight, the major difference being the extent of the degradation.

3.6 The effect of tritosomes on the release of MTX

3.6.1 Release of MTX at pH 5.6 in the presence and absence of tritosomes

A reaction mixture (7.5ml) was produced (HSA:MTX:ECDI = 1:58.3:86.5) in PBS pH7.4 and left for 26 hours at 25°C before being split into 5 x 1.5ml samples and frozen. One of these samples was then thawed and fractionated on a Sephadex G25 column (2.6 x 70cm) using pH5.6 buffer (McIlvaines) as the mobile phase; the more acidic conditions being chosen to mimic the lysosomal environment. The pH of the high molecular weight fraction collected in 25ml was measured at pH = 5.61 and its MCR was found to be 8.9.

The conjugate solution was filtered through a sterile 0.2um filter and then used to produce samples with and without tritosomes as follows:-

		Volume (ml)	
		sample (A)	sample(B)
1.	Conjugate in buffer (HSA = 0.416mg/ml)	1.00	2.00
2.	Reduced glutathione (73.5mM)	0.08	0.16
3.	EDTA (14.8mM)	0.08	0.16
4.	1% (w/v) Synperonic OP10 solution	0.03	0.06
5.	H_2O	0.01	0.02
6.	Tritosome solution	0.30	-
7.	Sucrose solution 2%w/v	-	0.60
	Total volume	1.50ml	3.00ml

200 ul samples of the mixtures were pipetted into 800ul vials and sealed; the vials were then incubated at 37°C for the appropriate length of time and then stored at -18°C prior to assay on the Superose 12 system (detection at 300nm).

The amounts of reduced glutathione and EDTA were selected to give a concentration of 5mM and 1mM respectively in the conjugate solution (ie 1 + 2 + 3) which equates to 3.9mM and 0.8mM overall. The surfactant solution (final concentration 0.02% w/v) was Synperonic OP10 and was at a sufficient level to clear the turbid tritosome solution inferring that the surfactant had solubilised the vesicle allowing release of the enzymes. The sucrose solution was added to blank B since this was the medium in which the tritosomes were isolated.

The amount of free MTX was calculated from the peak height compared to standards and expressed as a percentage (Table 3.4).

Table 3.4 Release of free MTX at pH 5.6 in the presence of tritosomes

Time	% free MTX determined by assay	
	Tritosomal Sample (A)	Blank (B)
Initial	0.0	0.0
1 hour	0.0	0.0
2 hours	0.05	0.0
4 hours	0.06	0.03
8 hours	0.18	0.06
16 hours	0.48	0.07
32 hours	0.80	0.12

3.6.2 Release of MTX in the presence and absence of tritosomes at pH 5.6 and pH 7.4

The release experiment was repeated using two separate reaction mixes (HSA:MTX:ECDI 1:58:87, PBS pH 7.4) which were left at room temperature for 22 hours before freezing prior to fractionation in pH5.6 and pH 7.4 buffers. The resulting MCR's were 9.9 ($\pm$0.3) and 9.3 ($\pm$0.2) respectively. The samples for incubation were produced as before with the exception that

higher levels of surfactant solution and sucrose solution were employed 10% and 20% w/v respectively. A blank was also produced which possessed conjugate at the same concentration as the other samples in buffer solution only.

The samples were incubated at 37°C for 0, 10 and/or 24 hours then frozen prior to assay using the TSK system (pH7.4). The peak heights of free MTX were measured and expressed as a percent of the total MTX; the results are means of duplicate injections of both sets of samples (Table 3.5).

Table 3.5 Release of free MTX in the presence of tritosomes

Sample incubation time (h) and pH		% free MTX (±sd) released		
		tritosomes	blank (surfactant)	blank (buffer)
pH 5.6	0	0	0	0
	10	1.24 (0.09)	-	0.77 (0.02)
	24	2.61 (0.15)	0.40 (0.02)	1.06 (0.08)
pH 7.4	0	0.80 (0.13)	0	0.34 (0.04)
	10	1.98 (0.18)	-	1.94 (0.06)
	24	2.52 (0.19)	0.84 (0.12)	2.61 (0.07)

3.7 The effect of tritosomes on the molecular weight distribution of the conjugates

The chromatograms from the experiment detailed in section 3.6 were also analysed using the molecular weight determination programme following subtraction of the relevant blank. The results are shown in Tables 3.6 and 3.7 and the former data are represented graphically in Figure 3.7.

The samples from 3.6.2 were also analysed for molecular weight distributions at 0, 10 and 24 hours using the calibrated TSK system. A tritosome blank was produced using the formula detailed in section 3.6.1 but replacing the conjugate with buffer alone (NB. surfactant 10% w/v and sucrose 20% w/v) this was also run on the TSK column and the results subtracted from the samples. The blank was found to have peaks eluting with the void volume and after about 14 minutes, this was probably due to the surfactants (eg Triton X-100) used to extract the tritosomes, the enzymes themselves and also the pH5.6 buffer used in the incubation mixture. The results are displayed in Table 3.8 and Figure 3.8. A typical example of a chromatogram from this experiment is shown in Figure 3.9.

The calculation of the overall molecular weight can be achieved using analysis B of the integration programme; this also yields information about the polydispersity of the samples (Table 3.9).

The results are means of two different samples except where indicated (standard deviations in brackets). A comparison of samples 1, 2 and 3 at pH7.4 shows that incubation at 37°C for 24 hours brought about a decrease

Table 3.6:
The molecular weight distribution of MTX-HSA conjugates in the presence of tritosomal enzymes in buffer solution (pH 5.6) at 37°C

	Peak No				
Incubation Time (Hours)	1 RT = 21.3	2 RT = 24.0	3 RT = c.30	4 RT = 33.8	5 MW <1500
Initial	22.6 (247)	65.5 (69.3)	8.9 (27.0)	1.3 (6.0)	-
1	19.2 (234)	64.5 (69.1)	10.0 (22.8)	6.3 (6.4)	-
2	19.6 (244)	69.1 (69.1)	8.4 (23.6)	2.9 (6.4)	-
4	16.5 (232)	56.7 (69.2)	12.5 (23.1)	6.6 (6.8)	7.6
8	11.0 (225)	51.8 (69.4)	12.5 (20.9)	7.7 (6.7)	16.9
16	6.2 (205)	38.9 (68.3)	13.3 (21.5)	8.9 (6.5)	27.2
32	5.4 (170)	23.9 (64.2)	15.2 (19.9)	10.2 (6.7)	38.8

Results expressed as % total area

MW x 1000 in parentheses
RT= Retention time (mins)
MW= Molecular Weight
HSA Concentration = 0.28 mg/ml

Table 3.7:
The molecular weight distribution of MTX-HSA conjugates in the absence of tritosomal enzymes in buffer solution (pH 5.6) at 37°C

	Peak No				
Incubation Time (Hours)	1 RT = 21.4	2 RT = 24.0	3 RT = c.30	4 RT = 33.6	5 MW <1500
Initial	22.0 (237)	63.7 (68.7)	7.2 (26.3)	2.5 (7.1)	4.5
1	21.5 (237)	59.7 (69.6)	9.8 (22.6)	7.7 (6.9)	1.2
2	19.5 (240)	59.0 (68.6)	9.3 (23.1)	5.2 (7.1)	3.1
8	22.0 (245)	60.2 (69.8)	9.4 (22.4)	6.9 (6.9)	1.4
16	20.3 (236)	59.1 (69.3)	8.9 (23.3)	7.7 (6.9)	4.0
32	20.6 (235)	56.6 (61.1)	9.9 (20.7)	6.5 (5.7)	6.4

Results expressed as % total area

MW x 1000 in parentheses
RT= Retention time (mins)
MW= Molecular Weight
HSA Concentration = 0.28 mg/ml

Fig.3.7 Effect of tritosomes on the molecular weight distribution of MTX-HSA conjugates (37 °C, buffer pH5.6)

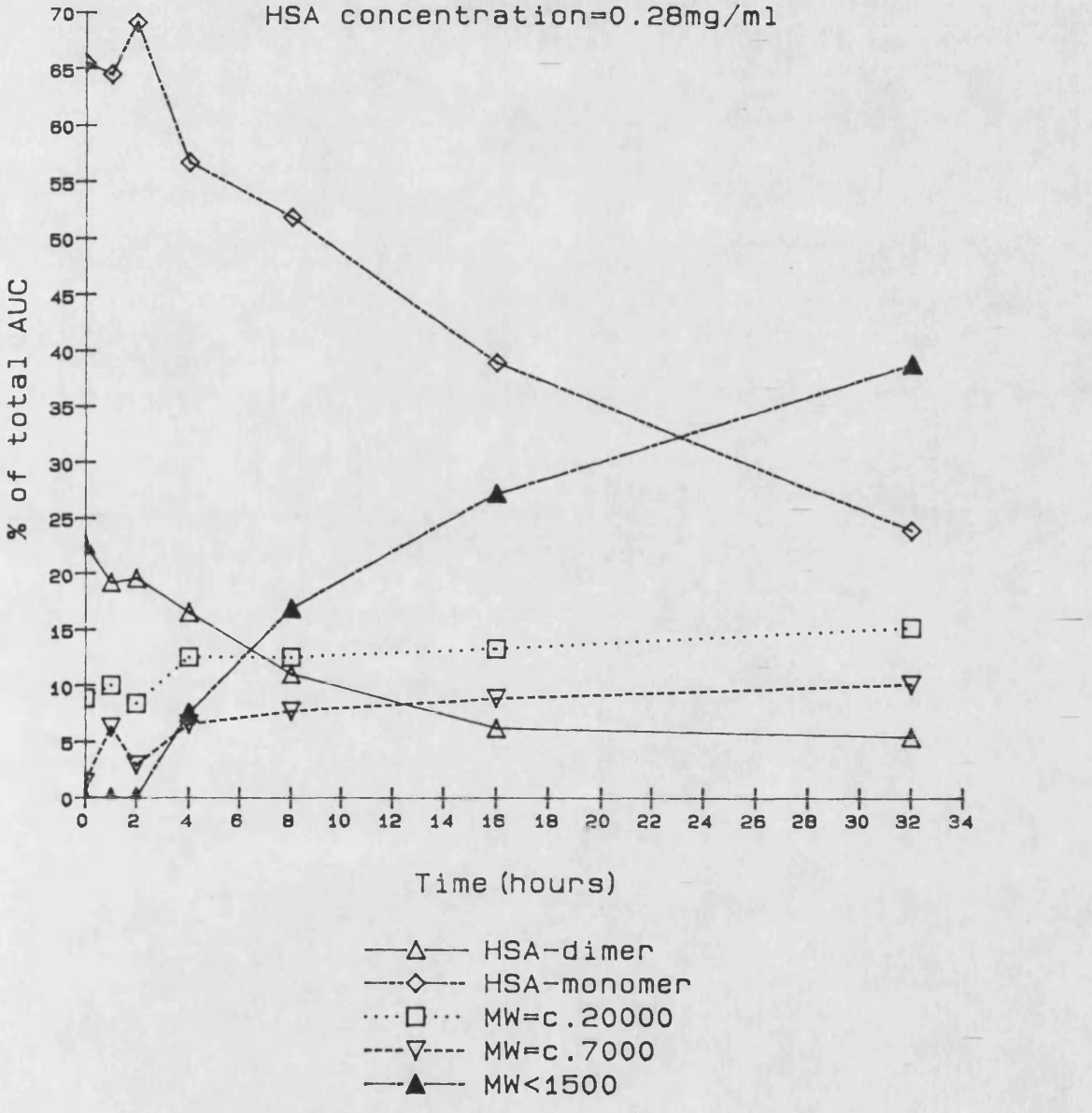


Table 3.8:
The molecular weight distribution of MTX-HSA conjugates in the presence and absence of tritosomes in buffer solutions (pH 5.6) at 37°C

	Molecular Weight					
Incubation Time	58,000	55,000	50,000	18,000	MTX	<1500
Initial[1]	97.7 (±0.8)	-	-	-	-	-
10 hours[1]*	-	64.0 (±3.0)	-	16.2 (±2.4)	5.6 (±0.1)	10.6 (±0.2)
24 hours[1]	-	-	40.2 (±2.8)	16.1 (±7.3)	8.8 (±1.8)	28.5 (±4.8)
Control pH 5.6 buffer only						
Initial[2]	98.3 (±0.2)	-	-	-	-	-
24 hours[2]	96.5 (±0.6)	-	-	-	1.0 (±0.2)	-

Results expressed as % of total area
(sd) n = 4 except * n = 2

1 = with tritosomes
2 = without tritosomes
HSA Concentration = 0.28 mg/ml

Fig.3.8 Effect of tritosomal enzymes on the molecular weight distribution of MTX-HSA conjugates in buffer solutions of different pH (37°C, 24 hours)

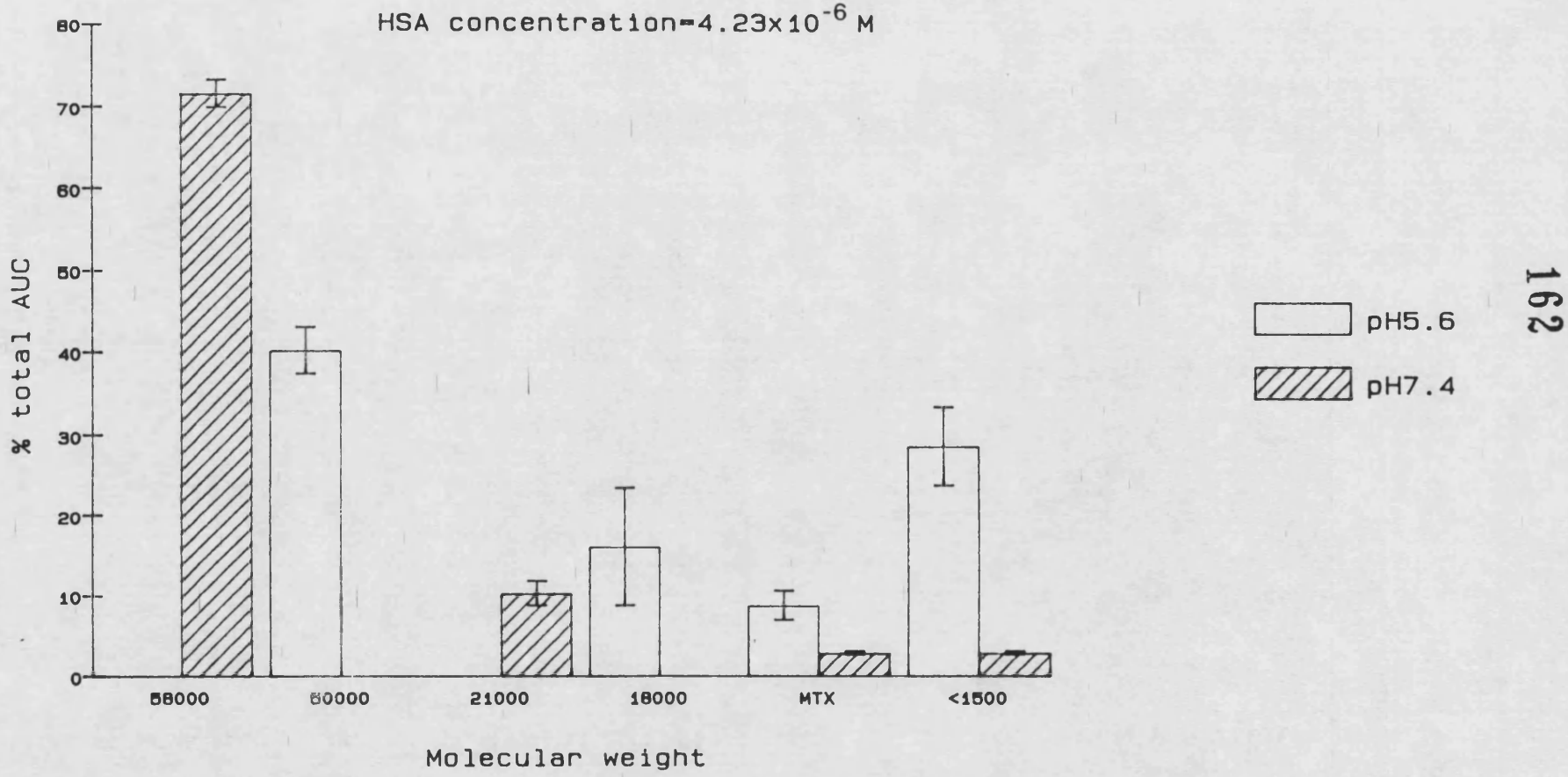


Fig 3.9

In vitro degradation of MTX_{10}-HSA conjugates at pH 5.6 and 37°C

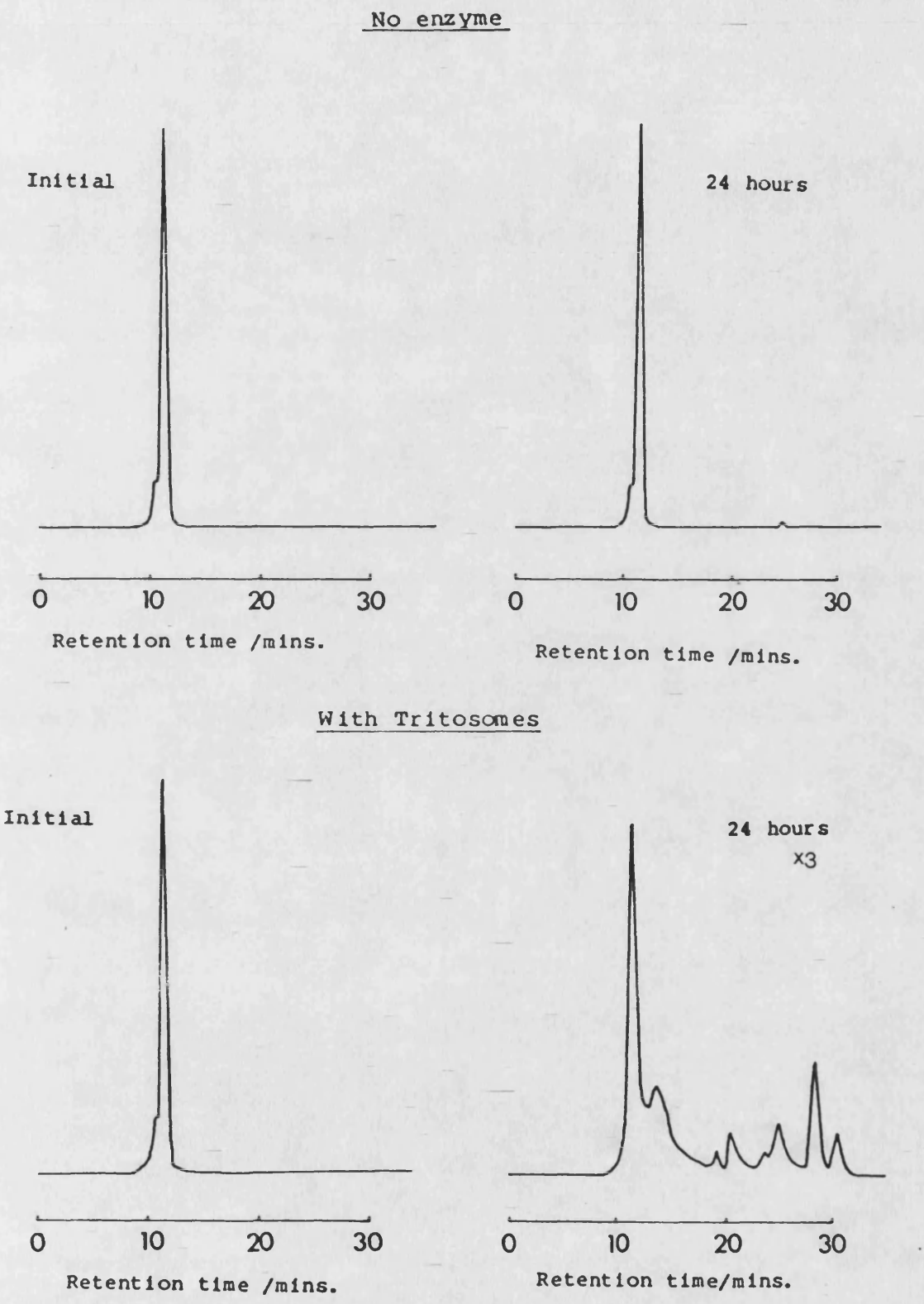


Table 3.9:
The overall molecular weight distribution of MTX-HSA conjugates, using a fixed baseline, in the presence and absence of enzymes in buffer solutions (pH 7.4 and 5.6) at 37°C

Sample	pH	Mw x 10^3	Mw/Mn
1-0H [1]	7.4	55.00	0.72*
1-24H [1]	7.4	39.24	8.55
2-0H	7.4	48.81 (±0.27)	2.96 (±1.37)
2-10H [1]	7.4	26.53	14.21
2-24H	7.4	24.38 (±0.81)	13.96 (±0.35)
3-0H	7.4	43.98	3.77
3-24H	7.4	16.40	12.04
4-0H	7.4	51.33 (±0.71)	2.77 (±0.75)
4-24H	7.4	49.61 (±0.11)	2.60 (±0.05)
6-0H	7.4	59.46 (±0.71)	3.75
6-24H	7.4	56.06 (±0.98)	6.78 (±0.87)
2-0H [1]	5.6	55.18	3.03
2-10H [1]	5.6	33.26	7.52
2-24H [3]	5.6	34.13 (±1.62)	7.31 (±4.04)
4-0H	5.6	52.42 (±0.49)	2.28
4-10H [1]	5.6	38.79	28.00
4-24H [3]	5.6	25.09 (±1.10)	60.03 (±12.40)
6-0H	5.6	57.07 (±0.41)	3.77 (±2.23)
6-24H [1]	5.6	55.72	3.80

* Below baseline
1 = Trypsin + sample 0.07:1 (Try:HSA)
2 = Trypsin + sample 0.73:1 (Try:HSA)
3 = Trypsin + sample 7.30:1 (Try:HSA)
4 = Sample + tritosomes
6 = Sample + buffer
0H = initial
10H = 10 hours at 37°C
24H = 24 hours at 37°C

Mw/Mn = polydispersity index
n = 2 except where indicated [n]
(±sd)

in molecular weight and an associated increase in the polydispersity. The higher the level of trypsin the greater the reduction in molecular weight. The trypsin sample (2) at pH 5.6 also exhibited a reduction in molecular weight and increase in polydispersity; however this is not as pronounced as at pH7.4 confirming the greater efficiency of the enzyme at the higher pH. A similar pH dependency was observed with the tritosomal samples with very little change at pH7.4 but a significant reduction in molecular weight occurring at pH5.6. The large polydispersity index infers that the tritosomal enzymes are capable of more complete dismantling of the MTX-HSA molecule at pH5.6 than is trypsin at pH7.4.

The deviation of the initial molecular weight of the samples from that of conjugate in buffer alone (6) arises due to the interference from the enzymes themselves since the chromatograms have not been subtracted.

3.8 The effect of reduced glutathione on the tritosomal degradation of MTX-HSA conjugates

Conjugates (MCR = 10, HSA concentration 0.416mg/ml) were isolated and the tritosomal solutions produced as described previously (Section 3.6.1), except that the buffer used was citrate-phosphate at pH 5 and one of the samples lacked reduced glutathione. The samples were incubated at 37°C prior to analysis on the Superose 12 column. The results are displayed in Table 3.10 and were determined from chromatograms following subtraction of the tritosome blank.

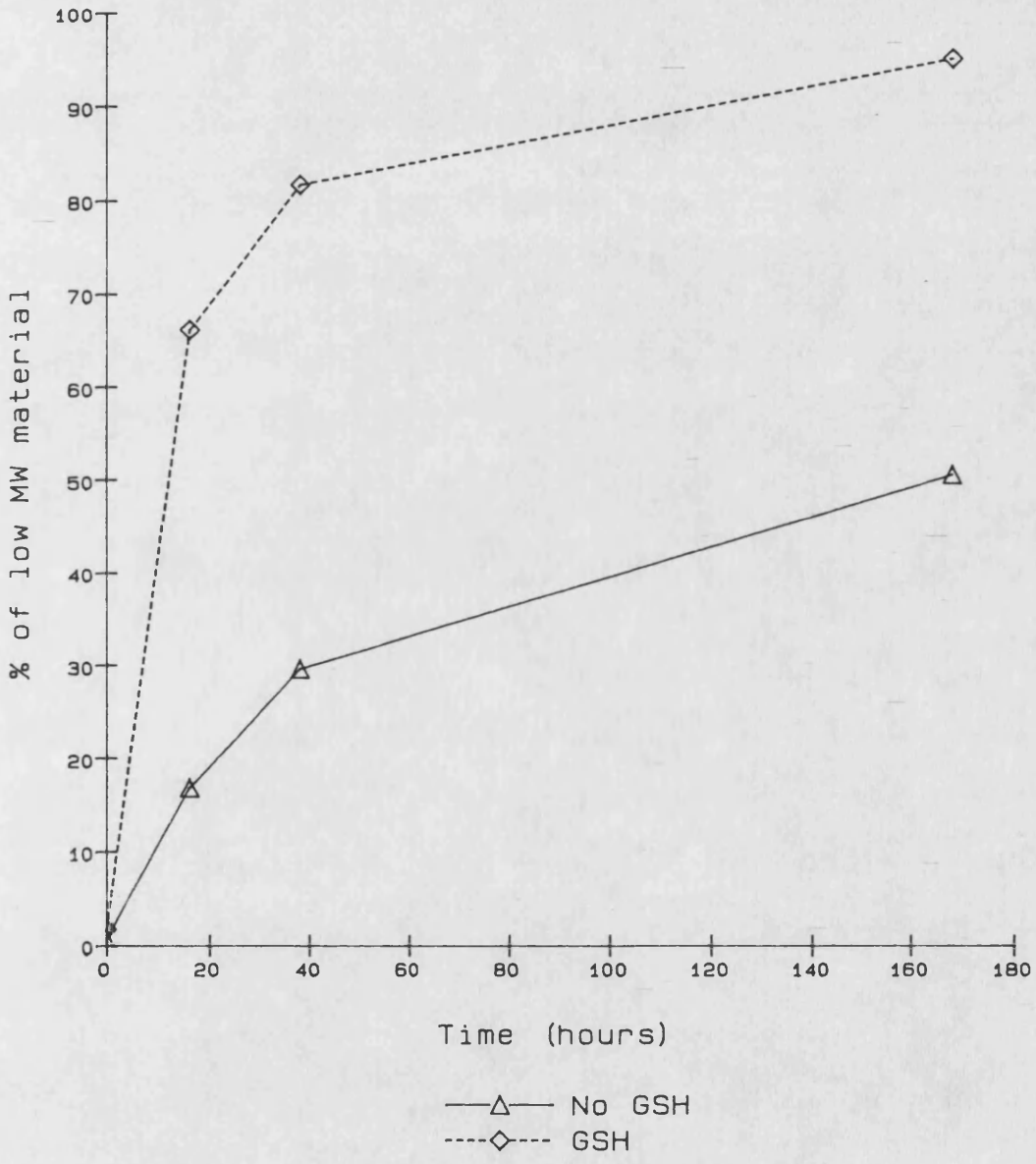
Table 3.10: The effect of reduced glutathione on the tritosomal degradation of MTX-HSA conjugates (37°C, pH 5)

	No GSH			GSH		
Peak No	1 Dimer	2 Monomer	5 MW<1500	1 Dimer	2 Monomer	5 MW<1500
Incubation Time						
Initial	7.9 (216)	88.5 (79.5)	0.7	7.8 (228)	86.7 (80.7)	1.7
16h	2.3 (142)	58.5 (71.2)	16.8	0.1 -	19.8 (58.9)	66.2
38h	1.6 (148)	46.3 (68.9)	29.6	-	4.3 (49.2)	81.7
7 day	- -	21.9 (61.3)	50.6	-	-	95.2

Results expressed as % of total area

MW = molecular weight x 1000 (in parentheses)
GSH = reduced glutathione

Fig.3.10 The tritosomal degradation of MTX-HSA conjugates in the presence and absence of reduced glutathione (in buffer pH5, 37°C)



3.9 Discussion

The addition of trypsin to the conjugate solution did not significantly affect the release profile of free MTX from the carrier; if anything the rate of release was slightly higher from the trypsinised samples. It is unlikely that the presence of trypsin would cause the release of more MTX since the enzyme cleaves peptide bonds on the carboxyl side of lysine or arginine residues within a polypeptide. Since the MTX is linked via an amide bond (section 3.2) the enzyme would not be expected to cause the release of free MTX. This result was further confirmed by an experiment where three different levels of trypsin were added to the same conjugate and release of free MTX at 37°C determined over a period of time; the resulting release profile (Figure 3.2) showed that all points could be fitted on the same curve. It is reasonable to conclude that there is no increase in the release of free MTX upon the addition of trypsin and that breakdown of the molecule into smaller units does not allow increased hydrolysis of the MTX peptide bond.

The addition of trypsin caused the HSA molecule to be broken down as was demonstrated by FPLC. The analysis of the degradation was problematical due to the interference of the trypsin molecule itself since the assay was at 279nm and at the high level of trypsin any absorbance was due mainly to the trypsin (AUC = 341% of control). This problem was alleviated to some extent by the use of the chromatogram subtraction programme which enabled subtraction of a trypsin control chromatogram (either initial or following incubation as appropriate) from an HSA/trypsin chromatogram. This permitted subsequent molecular weight analysis having reduced the AUC to 103% of that of the standard. In order to ensure that the results were

valid the data capture programme had to be initiated at precisely the same point for each sample. This was achieved by way of the autosampler which automatically started the programme when the injector valve was rotated. The efficiency of this method was confirmed by the reproducibility of the retention times of both HSA and MTX. The chromatograms for incubated enzyme show that at the higher level autodigestion occurred; very little breakdown was seen at the lower level although a 20% reduction in the area of the main peak was observed.

The addition of trypsin resulted in a reduction in the relative level of HSA (dimer and monomer, and an increase in lower molecular weight material at approximately 10, 20, 30 and 40,000 Daltons. The total amount of material below 1,500 Daltons was approximately 15% at the high level of trypsin after 24 hours although this tended to be as a broad distribution of material as opposed to specific peaks. The information presented (Table 3.3) demonstrated that trypsin was capable of dismantling the HSA molecule perhaps by way of intermediate molecular-weight polypeptides. An alternative explanation for the observed pattern of degradation is that there exist portions of the molecule that are more resistant to enzymatic degradation than are others. It was clear that the trypsin did not directly cleave the HSA into low molecular weight derivatives as could possibly have happened, if all sites were equally accessible, due to the fairly random distribution of lysine and arginine residues. This would have resulted in a broad distribution of low molecular weight material due to the variable length of peptides between lysine and arginine residues. It should be pointed out that the assay at 279nm will only detect those residues or bonds absorbing at that particular wavelength (essentially they must contain a tyrosine residue). Thus the profile obtained using

this method may not be a complete reflection of the true degradation products.

The effect of the autodigestion of trypsin on the resulting chromatogram is difficult to evaluate since the addition of substrate to enzyme in solution is likely to reduce the extent of autodigestion. Consequently at the higher enzyme concentration the subtraction of the chromatograms following incubation may have resulted in a distorted molecular weight distribution for degraded HSA. This did not pose a problem at lower enzyme levels since its effect on the chromatogram was minimal. If this was the case then part of the peak occurring at around MW 30,000 may have been due to trypsin and there could possibly have been a greater proportion of low molecular weight material released on digestion of HSA.

The trypsinisation of MTX-HSA conjugates brought about a similar dismantling of the macromolecule although the molecular weight fractions were not identical. In this case the assay was performed at 300nm thus the presence of the enzyme had very little effect on absorption and the assay was in fact monitoring peptide fragments with MTX as a marker. The presence of MTX on the HSA molecule did not appear to prevent the digestion of the macromolecule and if anything the rate of disappearance of the HSA monomer was more rapid than for unconjugated HSA with trypsin. The decrease in the area of the monomer peak was accompanied by a decrease in its apparent molecular weight. This was probably explained by removal of terminal oligopeptides from some conjugate molecules. Differing levels of trypsin resulted in different rates of degradation and appearance of low molecular weight material. The low molecular weight material (ie below MW c.1,500) included any free MTX and also material with a Kav

exceeding 1 implying some degree of column interaction similar to MTX. This was probably MTX-lysyl-oligopeptide derivatives.

The UV analyses of the fractions collected from a degradation run (section 3.4) all revealed the MTX type spectrum, however when the ratio of 370/280 was calculated it showed that some fractions contained proportionately more MTX in relation to the amount of peptide (or tyrosine) present. The apparent release of free MTX was higher when calculated by peak area as a percent of total area, rather than by assaying the MTX using the peak height of an MTX standard. This may be due to slight shift in λ max encountered upon conjugation although it is more likely to have been due to the method of calculating peak area (by dropping a vertical to the baseline). This would have resulted in a higher value since the MTX peak was not completely resolved from the previous peak. The peak height method was therefore used to determine the release of free MTX as for the non-enzymatically degraded samples. The change in MW of the dimer/monomer was not observed in the absence of trypsin, the only alteration in the chromatogram being a drop in the percentage of each which was due to the release of free MTX.

The degradation of MTX-HSA conjugates appeared to proceed via an intermediate molecular weight fraction of approximately 20,000 Daltons which formed fairly rapidly and then slowly disappeared as more lower molecular weight material was formed. This suggests that the molecule was cleaved to form primary degradation products and then cleaved again, as opposed to the small peptides being cleaved off the macromolecule directly. The latter would be unlikely due to the globular nature of the protein.

The addition of trypsin at the lower pH brought about the breakdown of the conjugate in a similar manner (ie the same molecular weight fractions appeared) to that observed at the higher pH but the rate of degradation was much slower ie following 24 hours at 37°C (Figure 3.5), 61% of the HSA peak remained at pH5.6 whereas only 35% remained at pH7.4. The molecular weight of the HSA peak was also found to have reduced from 59,000 to 52,000. The amount of low molecular weight material was significantly less at the low pH (2% as opposed to 17% at pH7.4) however the percentage at around 23,000 was approximately the same (21 and 24%). This difference in activity was expected since the pH optimum for trypsin is between 7 and 9. The decreased release of free MTX at reduced pH was confirmed in the experiment where only 1% of free MTX was released after 24 hours at pH5.6 whereas 3.4% were released at the higher pH (using MTX_{10}-HSA). This may have been due to the different buffer systems (ionic strengths were the same) used however earlier experiments employed all phosphate buffer systems and showed similar results.

The degradation of MTX-HSA conjugates using tritosomes was performed with enzymes extracted from rat liver. The tritosomes used were from two production batches as described in Appendix A3 and were stored at -18°C prior to use. Tritosomes were tested for activity (Appendix A3) using a standard method. The surfactant selected (Synperonic OP10) to solubilise the lysosomal membrane was similar to Triton X-100 routinely used by other workers and was used since it was least likely to interfere with the assay (ref. Appendix A3). Reduced glutathione was added to the digestion mixture to activate thiol proteases and EDTA was present in order to chelate any metal ions that would interfere with enzyme activity. The

tritosome blank was run on the column (detection at 300nm) and the resulting chromatogram showed two peaks, one eluting with the void volume and the other corresponding to an apparent molecular weight of around 18,000. The first peak was thought to be due to particulate surfactant or membrane components and the second was due to the dissolved components in the buffer. The blank chromatograms analysed were subtracted from the degraded conjugate chromatograms in order to produce results for the degraded MTX-HSA alone (manual subtraction was occasionally necessary due to the unexplained variability in the area of the second peak of the blank).

The effect of tritosomes on HSA alone was not investigated due to the high level of absorbance of the tritosomes at 280nm which would probably have had the effect of masking any degradation of the macromolecule (even using the subtraction programme). Degradation experiments were performed using conjugates by analysing the eluant at 300nm which minimised the absorbance of materials other than MTX (the tritosome system contributing to about 25% of the total area).

The release of free MTX from the conjugate (MCR = 9) in the tritosomal sample (at pH5.6) was found to be less than 1% after 32 hours at 37°C, in contrast the blank sample (B) (ie. no tritosomes) was found only to release 0.1%, which was much lower than previously demonstrated at this pH. It is thought that this may have been due to the presence of surfactant. Analysis of the molecular weight distribution (Table 3.6) over an incubation period of 32 hours at 37°C reveals that there was a reduction in both the amount and the molecular weight of the dimer and monomer peaks of the conjugate from 66% to 24% in the case of the monomer

(MW 69,000 to 64,000). The initial sample was found to contain some other low MW material at 27,000 and 6,000 which would have been caused by digestion of the conjugate prior to freezing and also upon thawing before analysis; the initial thus is in fact an intermediate time between 0 and 1 hour. The incubated samples developed peaks corresponding to around 20,000 and 6,000 Daltons which slowly increased to levels of 15 and 10% respectively. After 32 hours incubation the majority of the material was found to be below MW 1,500 (39%) some of which eluted at the retention time of MTX and some having a longer retention time, presumably due to interaction with the column. The blank sample showed only a slight drop in the levels of dimer and monomer (86 to 78% overall) with very little change in the molecular weight (especially up to 16 hours). The samples did however demonstrate the presence of peaks at around 20,000 and 6,000; these were present at a constant level throughout and may have been due to the buffer or additives to the conjugate solution. There was also some material present at longer retention times ie. 1.2 - 6.4% showing no definite increase in peak area.

The effect of tritosomes was further investigated using duplicate samples (MCR = 10) fractionated in pH5.6 and pH7.4 buffer (Figure 3.8). The release of free MTX was found to be lower at the more acidic condition (1.1% release in 24 hours at pH5.6, 2.6% at pH7.4) the relatively low level of release in buffer at pH7.4 was probably due to the method of preparation. The incorporation of surfactant with the buffer reduced the release of MTX to 0.4% and 0.8% at pH5.6 and 7.4 respectively. The addition of tritosomes had no effect on the release of drug at the elevated pH but brought about a quite substantial increase at the lower pH (2.6% in 24 hours).

The addition of tritosomes had a significant effect on the MW distribution at pH5.6 with a reduction in the main peak to 40% after 24 hours and a drop in MW from 58,000 to 50,000, this was associated with 29% of the material being below about 1,500 and 16% at around 18,000. In contrast the only effect on the conjugate peak in buffer pH5.6 alone was a slight reduction to 97% over 24 hours with the appearance of 1% free MTX. The 24 hour digestion with tritosomes at pH7.4 exhibited much reduced activity with 80% of the main peak remaining (with no significant change in the molecular weight) and only abut 10% appearing below MW 1,500.

The effect of pH on the activity of the enzymes is further exemplified by the comparison of the overall molecular weight distributions (Table 3.9) and polydispersity indeces. The increasing level of trypsin (24 hour at pH7.4) resulted in a decrease in the MW with the polydispersity index being of the same order of magnitude. This implies that the higher level of trypsin resulted in a more extensive dismantling of the conjugate. A reduction in the pH to 5.6 also caused reduced efficiency. The control samples in buffer alone show little change in the molecular weight and polydispersity index; the slight increase occurring at pH7.4 may have been due to the free MTX peak. The tritosome samples exhibited a large difference between pH conditions with virtually no change in MW or index at pH7.4 inferring that the enzyme system was inhibited in alkaline conditions (as is the case when NH_4Cl is added to <u>in vitro</u> systems). The change in polydispersity index was however marked at pH5.6 where it increased from its initial value of 2.3 to 60.0 after 24 hours at 37°C with an associated drop in MW. This infers that the distribution is far from being monodispersed and reflects the situation where little material

of intermediate molecular weight is formed, it being of either the high molecular weight of the conjugate or below 1,500.

The high degree of polydispersity with the tritosome samples was due to the panoply of enzymes capable of hydrolysing a variety of bonds and thus able to break the conjugate down into a series of oligopeptides with only a few amino acids. This may also account for the increased release of free MTX in the presence of tritosomes due to carboxypeptidases. The trypsin on the other hand is specific for lysine/arginine bonds and although there is a fairly random distribution throughout HSA some bonds will be more susceptible than other leading to certain high molecular weight fragments. Trypsin may not be able to degrade lysine with MTX attached since the amino group will be unprotonated and will have the bulky drug molecule instead.

The partial thiol dependent nature of the tritosomal degradation of MTX-HSA was demonstrated by the omission of reduced glutathione (section 3.8) which showed a slower rate of degradation of the monomer peak than the sample with reduced GSH present. In the latter case the MTX-HSA had been completely degraded following 7 days at 37°C whilst 22% was still present as the high molecular weight conjugate in the absence of GSH. This experiment exhibited greater activity than previous runs (eg section 3.6) which may have been due to the fact that a lower pH buffer was used in this case or that a different batch of enzymes were used which had lower activity. Unfortunately the activity of the first batch of tritosomes was not determined. Some of the fragments produced may not have had MTX attached to them and thus would not have been detected readily (due to a lower extinction coefficient) causing a distorted distribution.

However the most important aspect with respect to the practical use of such prodrugs, is whether or not the MTX was released linked to oligopeptides of a low molecular weight which would be able to leave the lysosome and would be capable of binding to DHFR and exerting cytotoxic action. The results of the above experiments suggest that such low molecular weight oligopeptide derivatives of MTX are extensively released in the presence of lysosomal enzymes.

CHAPTER FOUR: SYNTHESIS, CHARACTERISATION AND DEGRADATION OF MTX-COPOLY(AMINO ACID) CONJUGATES

4.1 Introduction

Synthetic peptides were originally produced in order to evaluate their chemical and physical properties as model proteins; they were also used as substrates to evaluate enzyme function and specificity. Synthetic polypeptides have proven useful in the analysis of the molecular conformation of proteins (Fasman, 1967). The synthesis, chemical and biological properties have been reviewed previously (Katchalski and Sela, 1958, Sela and Katchalski, 1959). In latter years polyamino acids have been used as carrier systems for cytotoxic drugs whereas smaller peptides have been synthesized to produce pharmacologically active compounds.

The copolymers produced in this study were synthesized using Leuchs anhydrides of the appropriate amino acids (lysine and glutamic acid). The methods used were adapted from Bergmann et al (1935), Hanby et al (1950) and Katchalski (1957). The ϵ-amino groups of L-lysine were protected by the benzoxycarbonyl group and the γ-carboxyl groups of the L-glutamic acid were protected using the benzyl ester, to prevent the occurrence of side reactions. The N-carbobenzoxy anhydride (NCA) derivatives were prepared by reaction of the N-carbobenzoxyl derivative (of the respective α-amino group) with phosphorus pentachloride; the cyclic anhydrides being formed via the acyl chloride intermediate. The polymerisation of the NCA's in DMF was initiated using a secondary amine (the mechanism is shown in Figures 4.3 and 4.4 (from Katchalski and Sela, 1958)) and subsequently deprotection of the product was achieved using hydrogen bromide in glacial acetic acid. This method has been reported to yield poly(amino acid)s retaining the L-configuration.

4.2 Synthesis of poly(amino acid)s

4.2.1 Preparation of N,N'-Dicarbobenzoxy-L-lysine

Lysine hydrochloride (10.0g; 0.055m) (I) was dissolved in 2N NaOH (82ml) in a 250ml reaction vessel and chilled on an ice bath. 25ml of chilled benzyl chloroformate (30g; 0.173m) and 4N NaOH (68.5ml) were added dropwise from two dropping funnels, with vigorous stirring, over a 30 minute period maintaining the temperature below 10°C and alkaline conditions. The mixture was then left stirring for a further 20 minutes before extraction with ether and then acidified with 5N HCl, maintaining the temperature below 10°C. The resulting oil was extracted into ethyl acetate which was dried over anhydrous magnesium sulphate then filtered, washed with 50ml of distilled water, separated and redried over anhydrous magnesium sulphate. The filtrate was distilled under vacuum (30 - 40°C; 20-25mm Hg) then 10ml of ether were added and evaporated off leaving a straw coloured syrup. The syrup was saturated with petroleum ether and left for 60 hours at room temperature; the resulting white solid N,N'-dicarbobenzoxy-L-lysine (II) was then filtered and dried under vacuum (12.56g; 55%) mp 77-79°C. (Ref. Scheme 4.1).

^{1}H NMR. 9.6(s)1H, 7.3(s)10H, 6.4(d)1H, 5.8(s)1H, 5.1(s)4H, 4.5(br s)1H, 3.1(s)2H, 1.7(m)2H, 1.4(m)4H. On deuteration peaks at 9.6, 6.4 and 5.8 ppm disappeared.

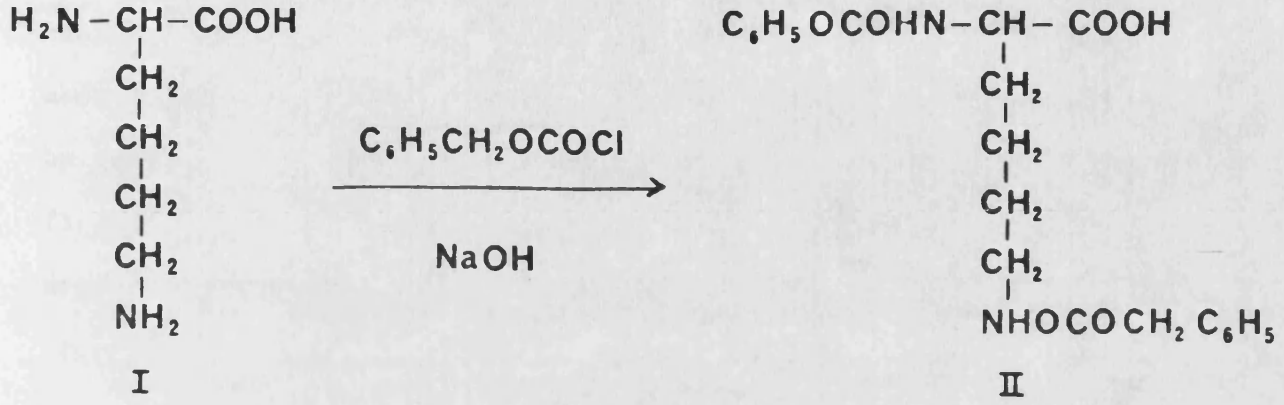
4.2.2 Preparation of ϵ,N-carbobenzoxy- α,N-carboxy-L-lysine anhydride

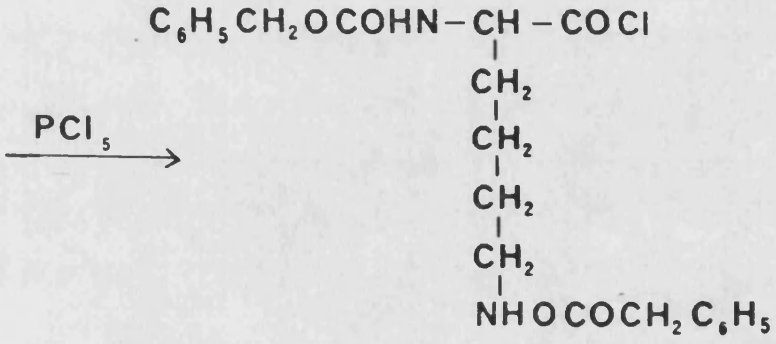
All glassware was dried before use at 105°C.

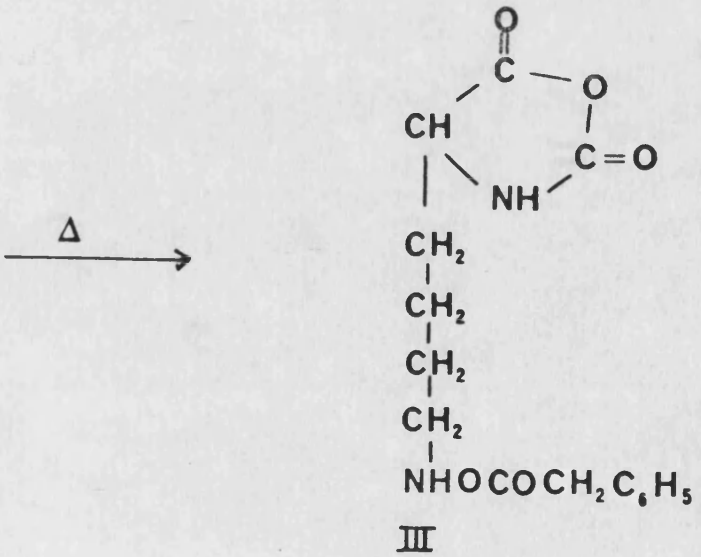
N,N'-dicarbobenzoxy-L-lysine (9.6g; 0.023m) (II) was dissolved in 100ml of dry ether and 20ml dry ethyl acetate with stirring on an acetone/ice bath. Phosphorus pentachloride (9.2g; 0.030m) was added and stirred vigorously for 10 minutes until most of the solid phosphorus pentachloride had gone into solution. The solution was quickly filtered and left at room temperature for 75 mins to precipitate. Dry petroleum ether was added to complete precipitation and the suspension was sealed and cooled in a refrigerator for 2 hours. The white precipitate was filtered and dried under vacuum over phosphorous pentoxide for 2-3 hours. The product was recrystallised eight times from warm dry ethyl acetate and dry petroleum ether. The pure white crystalline anhydride, ϵ,N-carbobenzoxy- α ,N-carboxy-L-lysine anhydride, (III), that resulted was dried under vacuum over phosphorus pentoxide (2.2g; 31%), mp 97-98°C with decomposition (Ref. Scheme 4.1).

^{1}H NMR 8.7(s)1H, 7.3(s)5H, 6.0(s)1H, 5.1(s)2H, 4.2(t)1H, 3.2(d)2H, 1.7(double m)2H, 1.5(s)4H. On deuteration peaks at 8.7 and 6.0 ppm disappeared.

Scheme 4.1 Preparation of ε,N-carbobenzoxy-α,N-carboxy-L-lysine anhydride







4.2.3 Preparation of γ-benzyl-L-glutamate

Glutamic acid (30.0g; 0.204m) (IV) was dissolved in a mixture of 200ml benzyl alcohol (209.2g; 1.937m) and 50ml of hydriodic acid (85.0g; 0.382m) and left at 25°C with occasional stirring. The product was precipitated by adding 400ml of absolute alcohol and 60ml of pyridine, this was then filtered and washed with absolute alcohol. The resulting white solid was dried at 35°C over silica gel then cooled over phosphorus pentoxide under vacuum.

The percentage of glutamic acid remaining in the precipitate was determined as 34% (by titrating an aqueous solution with 0.02N NaOH using bromothymol blue indicator) consequently the material was recrystallised from 400ml of hot water containing 6.8g of sodium bicarbonate (CO_2 evolved). The suspension was brought up to 90°C and sufficient water added to produce a solution which was then filtered through a warm sinter. Crystallisation was initiated by cooling; the temperature was reduced to 10°C then the product was filtered and rinsed with chilled water and dried over phosphorus pentoxide under vacuum. The product γ-benzyl-L-glutamate (V) was in the form of white crystalline plates (18.4g; 80%) mp 169°C (Ref. Scheme 4.2).

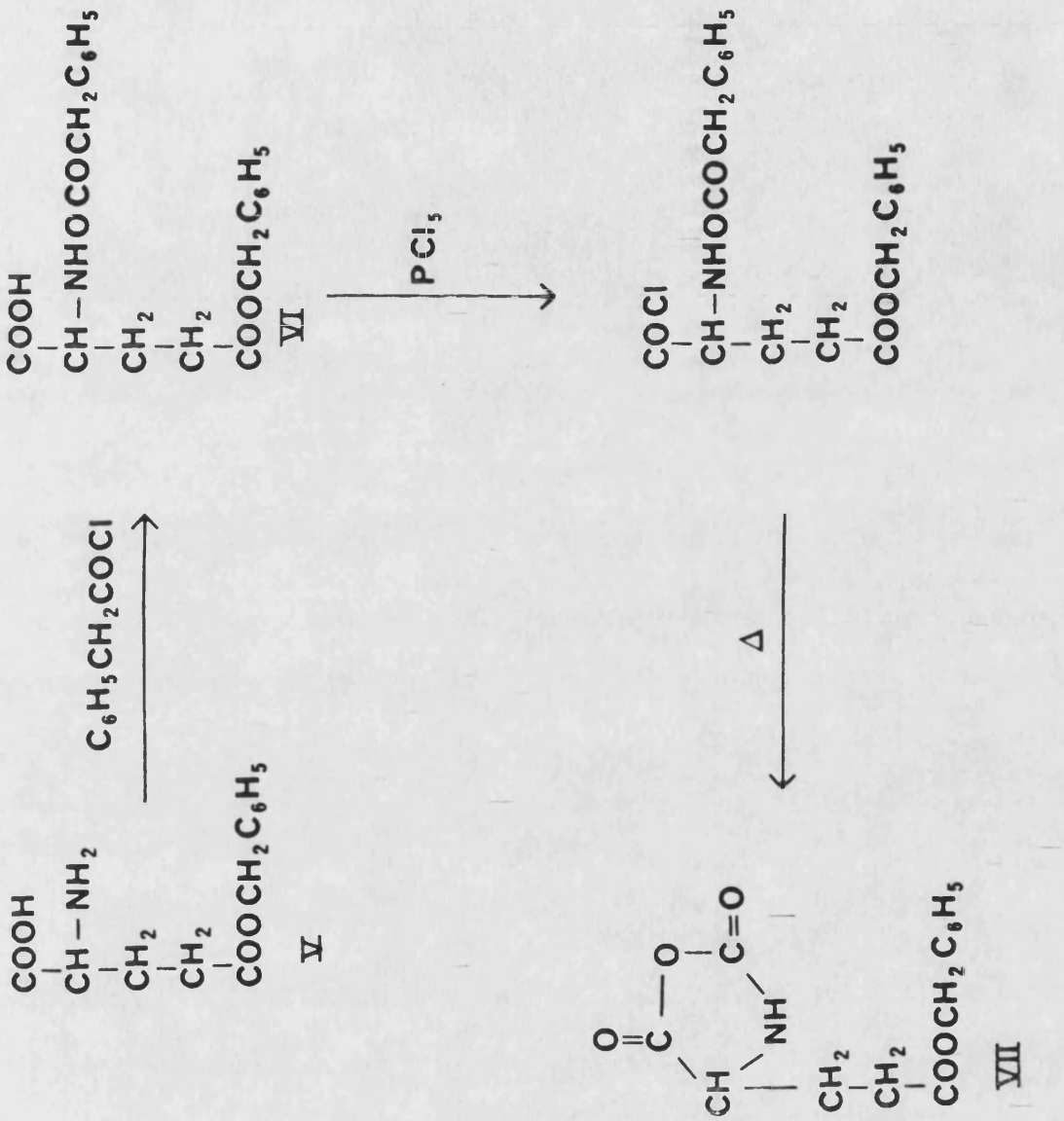
^1H NMR 7.5(s)5H, 5.2(s)2H, 3.8(t)1H, 2.6(t)2H, 2.1(q)2H.

4.2.4 Preparation of γ-benzyl N-carbobenzoxy-L-glutamate

γ-benzyl-L-glutamate (V) (17.8g; 0.075m) and sodium bicarbonate (15.0g; 0.179m) were added to 750ml of distilled water and heated to 70°C to form a solution (with stirring). 15ml of benzyl chloroformate (18.0g; 0.104m) were added with vigorous stirring (CO_2 evolved), the reaction was allowed to proceed for 45 mins (temperature dropped to 50°C) then it was cooled on ice. The mixture was then extracted with ether and acidified with 5N HCL to pH<3 and the resulting oil extracted into ether; this was then washed with 500ml of water containing 5.4ml of 1.4N potassium bicarbonate and then with 350ml of water. The ethereal solution was dried over anhydrous magnesium sulphate, filtered and then distilled under vacuum at 40°C. Cooling precipitated a white solid from the yellow syrup. 900ml of carbon tetrachloride were added, warmed to 50°C and distilled under vacuum to a volume of 100-150ml and left under ambient conditions overnight; islands of monoclinic crystals were formed. The volume was reduced by 10-20ml and then the mixture was refrigerated prior to filtration and washing with chilled carbon tetrachloride. The product γ-benzyl N-carbobenzoxy-L-glutamate (VI) was dried under vacuum over phosphorus pentachloride (13.9g ; 50%)mp 75-76°C (Ref. Scheme 4.2).

^1H NMR. 10.5(s)1h, 7.3(s)10H, 5.1(s)4H, 4.4(d)1H, 2.5(m)2H, 2.0(2m)2H. On deuteration peaks at 10.5 and 6.7 ppm disappeared.

Scheme 4.2 Preparation of γ-benzyl N-carboxy-L-glutamate anhydride



4.2.5 Preparation of γ-benzyl N-carboxy-L-glutamate anhydride

All glassware was dried at 105°C and cooled over silica gel

γ-benzyl N-carbobenzoxy-L-glutamate (14.0g; 0.038m) (VI) were dissolved in 85ml dry ether and then cooled on an acetone/ice bath. Phosphorous pentachloride (9.4g; 0.045m) were added and stirred at room temperature for 10 minutes until most of the solid had gone into solution when it was quickly filtered and chilled to 0°C to complete the precipitation. The product was recrystallised three times from dry ethyl acetate and dry petroleum ether, with the careful exclusion of moisture, then dried at 35°C under vacuum and cooled under vacuum over phosphorus pentoxide. The pure γ-benzyl N-carboxy-L-glutamate anhydride (VII) was in the form of white needle-like crystals (3.8g; 38%) mp 93-95°C (sealed) with decomposition (96-97°C reported) (Ref. Scheme 4.2).

^{1}H NMR. 8.3(s)1H, 7.3(s)5H, 5.1(s)2H, 4.4(t)1H, 2.6(t)2H, 2.1(m)2H. On deuteration peak at 8.3 ppm disappeared.

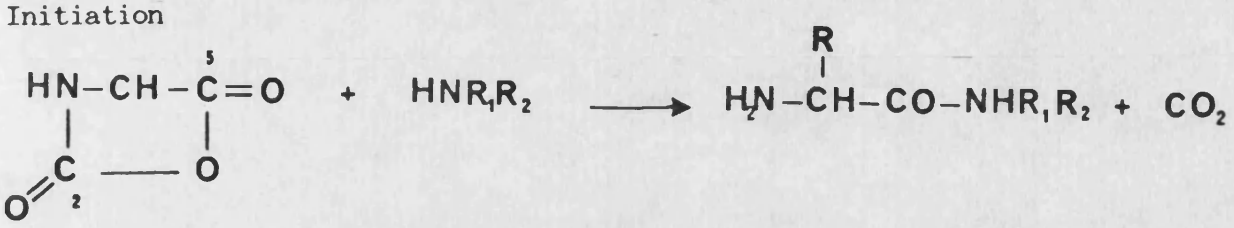
4.2.6 Preparation of ε,N-carbobenzoxy-L-lysine- γ-benzyl-L-glutamate copolymer

ε,N-carbobenzoxy- γ-N-carboxy-L-lysine anhydride (CLA) (III) (999.2mg; 3.26 x 10^{-3}m) and γ-benzyl N-carboxy-L-glutamate anhydride (BGA) (VII) (863.5mg; 3.28 x 10^{-3}m) were dissolved in redistilled dry dimethylformamide (35ml) to form a 5.3% w/v solution. The reaction was initiated by adding 77.5ul of a solution (4.34%v/v) of diethylamine in DMF

Scheme 4.3

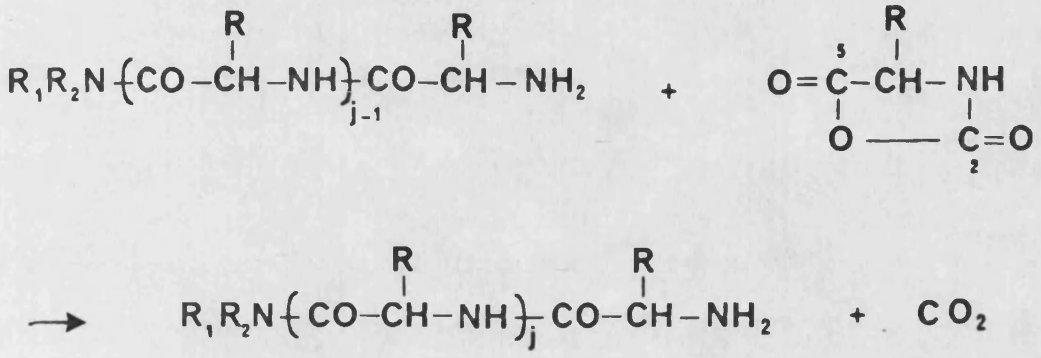
Polymerisation of N-carboxyanhydrides with primary and secondary amines

Initiation

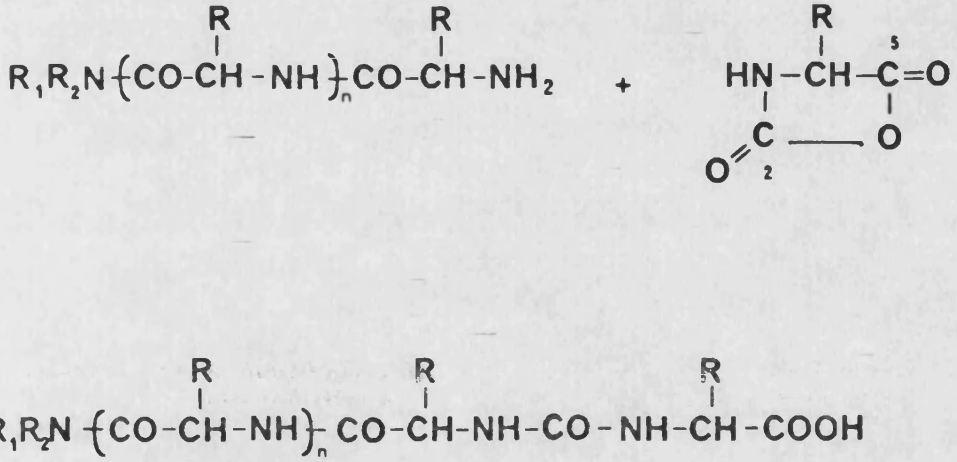


Amide formation following the reaction of the amine with the 5 carbonyl of the NCA

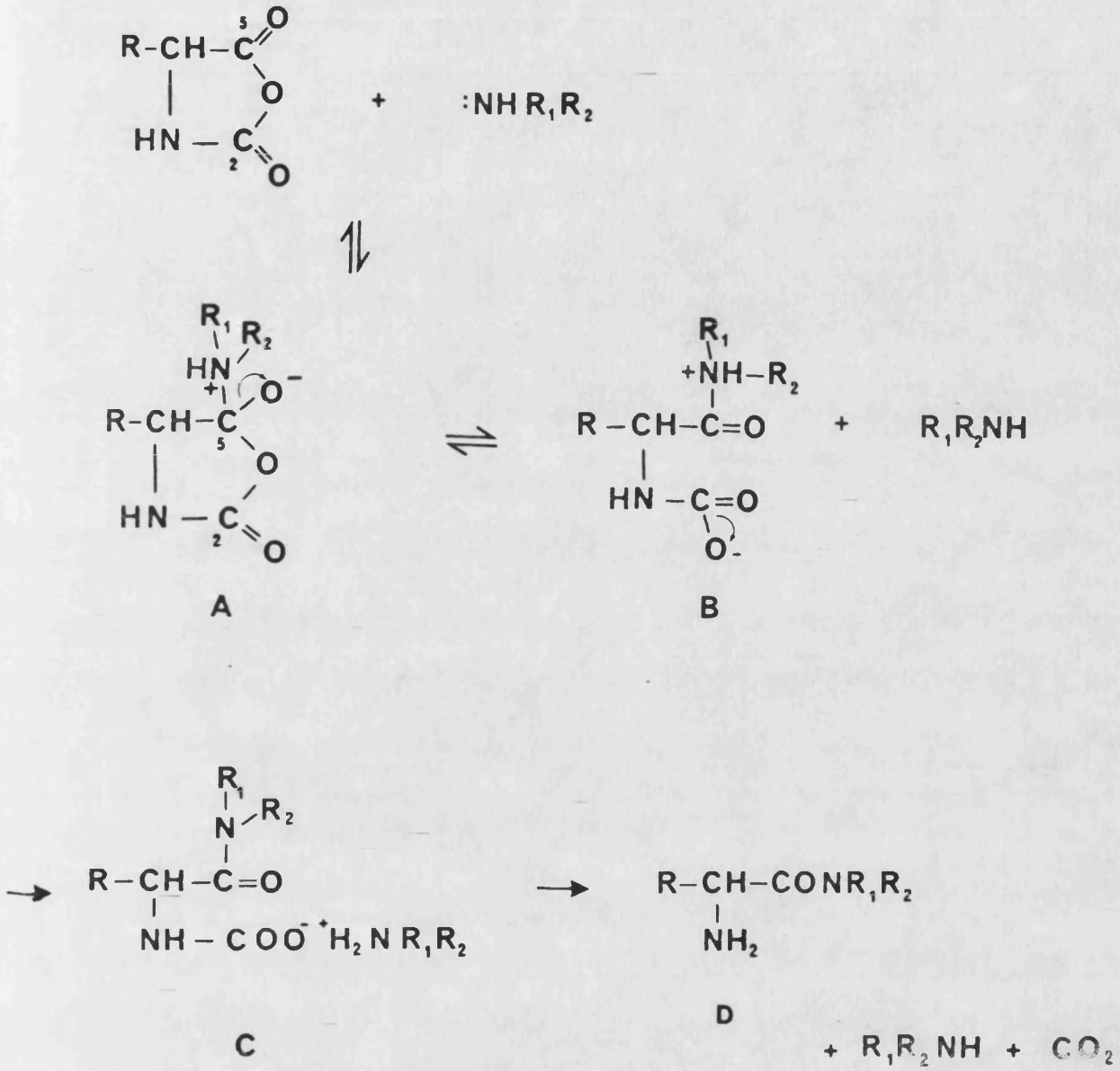
Propogation



Termination



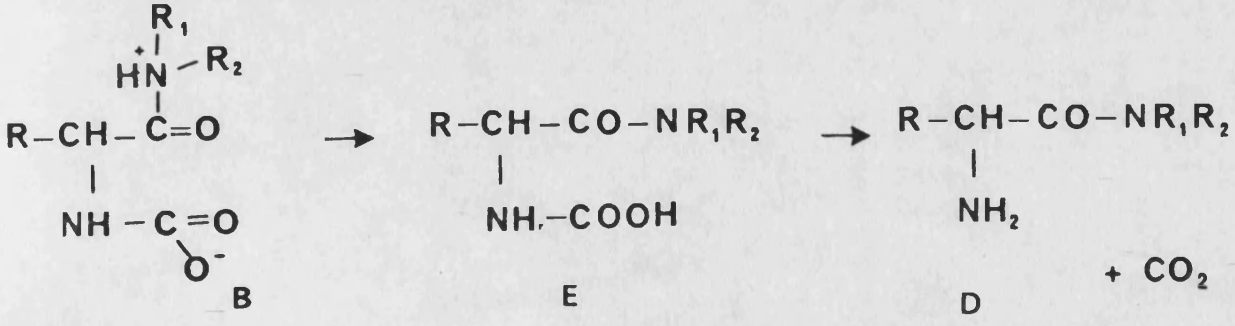
Scheme 4.4 Reaction mechanism



A = reversible intermediate formed between nucleophilic amine and electrophilic carbon (5) of the oxazolidine-2-5-dione.

A forms the zwitterion B and in excess amine forms the ammonium carbamate (C). The ammonium salts of α-amino acid ester carbamates readily split off carbon dioxide at room temperature to form the amino amide (D).

The above assumes proton donation by B to the nucleophilic amine; however the carboxylate group of B may accept a proton.

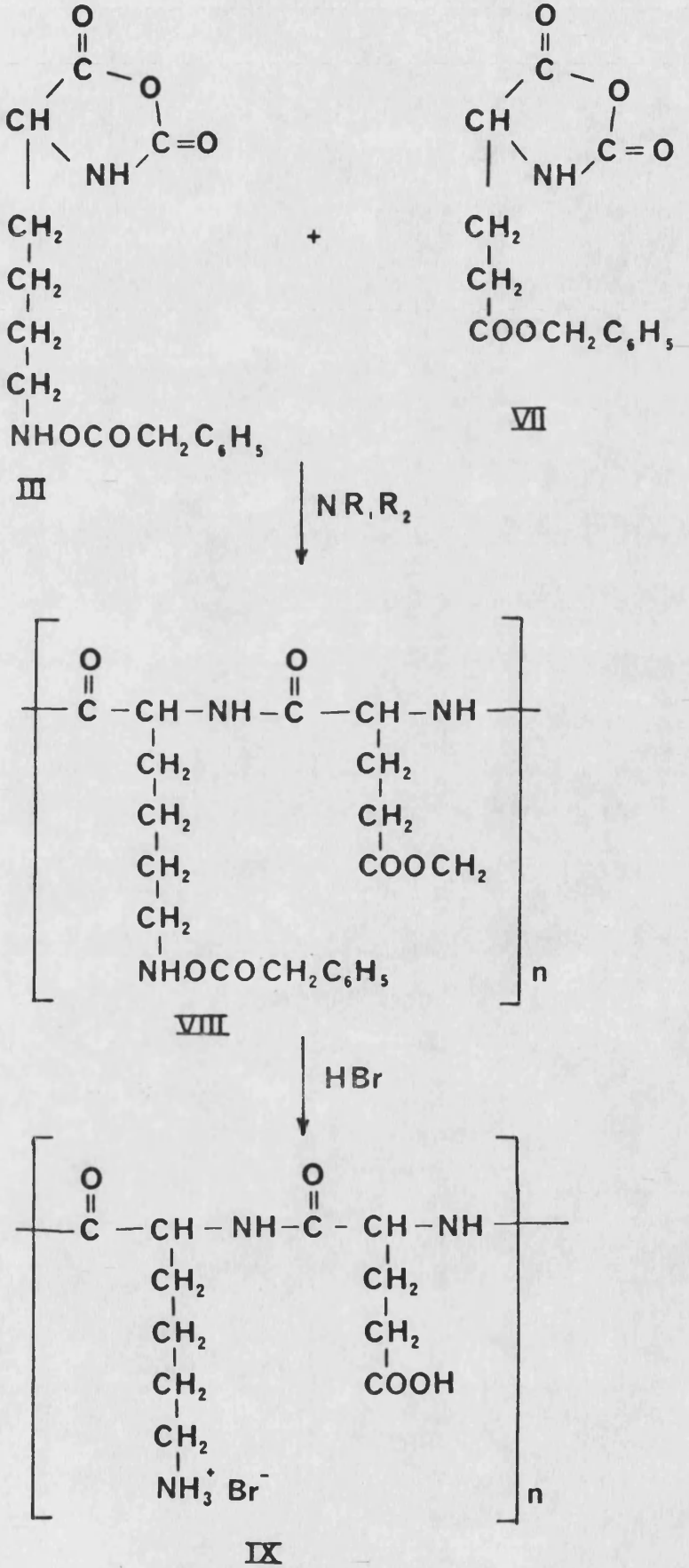


(3.17×10^{-5}m) giving an anhydride to initiator ratio (A/I) of 206:1. The flask was sealed and left in the dark under ambient conditions for 72 hours and then transferred to a 30°C oven until termination. A sample was removed after 142 hours, diluted with DMF and the intrinsic viscosity determined (to give an indication of the degree of polymerisation). The reaction was terminated after about 145 hours by the addition of 100ul 0.05N HCl. The white precipitate was left at ambient overnight then filtered and recrystallised from chloroform and petroleum ether before drying under vacuum over phosphorus pentoxide. The ε,N-carbobenzoxy-L-lysine-γ-benzyl-L-glutamate copolymer (VIII) was isolated as a white solid (1.33g; 72%). (Repeat 85%) (Ref. Scheme 4.5).

4.2.7 Preparation of L-lysine-L-glutamate copolymer

ε,N-carbobenzoxy-L-lysine-γ-benzyl-L-glutamate copolymer (VIII) (0.21g) was placed in a conical flask with a silica gel side arm and 5ml of a 33%w/w solution of hydrogen bromide in glacial acetic acid were added; a clear yellow solution was formed accompanied by the evolution of gas. Precipitation started to occur and then the solution was warmed to 50°C, then cooled and precipitation completed by the addition of anhydrous ether. The material was then extracted several times with anhydrous ether until the supernatant was clear. The residue was then dried under vacuum. The residue was then dissolved in a few drops of distilled water and then sufficient absolute alcohol was added to form a clear solution, the polymer was then precipitated as a white plastic material by the addition of ether. The supernatant was decanted off and the product dried under vacuum over silica gel. The yield of L-lysine-L-glutamate copolymer (IX) was 0.11g (73%) (Ref. Scheme 4.5).

Scheme 4.5 Preparation of L-lysine-L-glutamate copolymer



4.2.8 Polymerisation of CLA and BGA in DMF using diethylamine as initiator

The polymerisation reaction obviously should produce an increase in molecular weight which would result in an increased solution viscosity. The viscosity of the reaction mix was determined using a suspended level viscometer (PSL No1) which had been soaked in chromic acid then washed and rinsed with acetone and dried. The viscometer was immersed in a viscometer bath at 25°C ± 0.1°C and flow times determined by stopwatch. The solvent used was redistilled DMF which was stored over a molecular sieve; all solutions were filtered (0.5um PTFE) into the viscometer and allowed to equilibrate before measurement.

Viscosity Calculations

Kinematic viscosity = viscometer constant (k) x flow time (sec)

η_o = viscosity of pure solvent

η = viscosity of solution

η rel = relative viscosity = η / η_o

= t / to

= flow time solution/solvent

η sp = η rel - 1 = specific increase in viscosity

η sp/c = reduced specific viscosity, where c = concentration

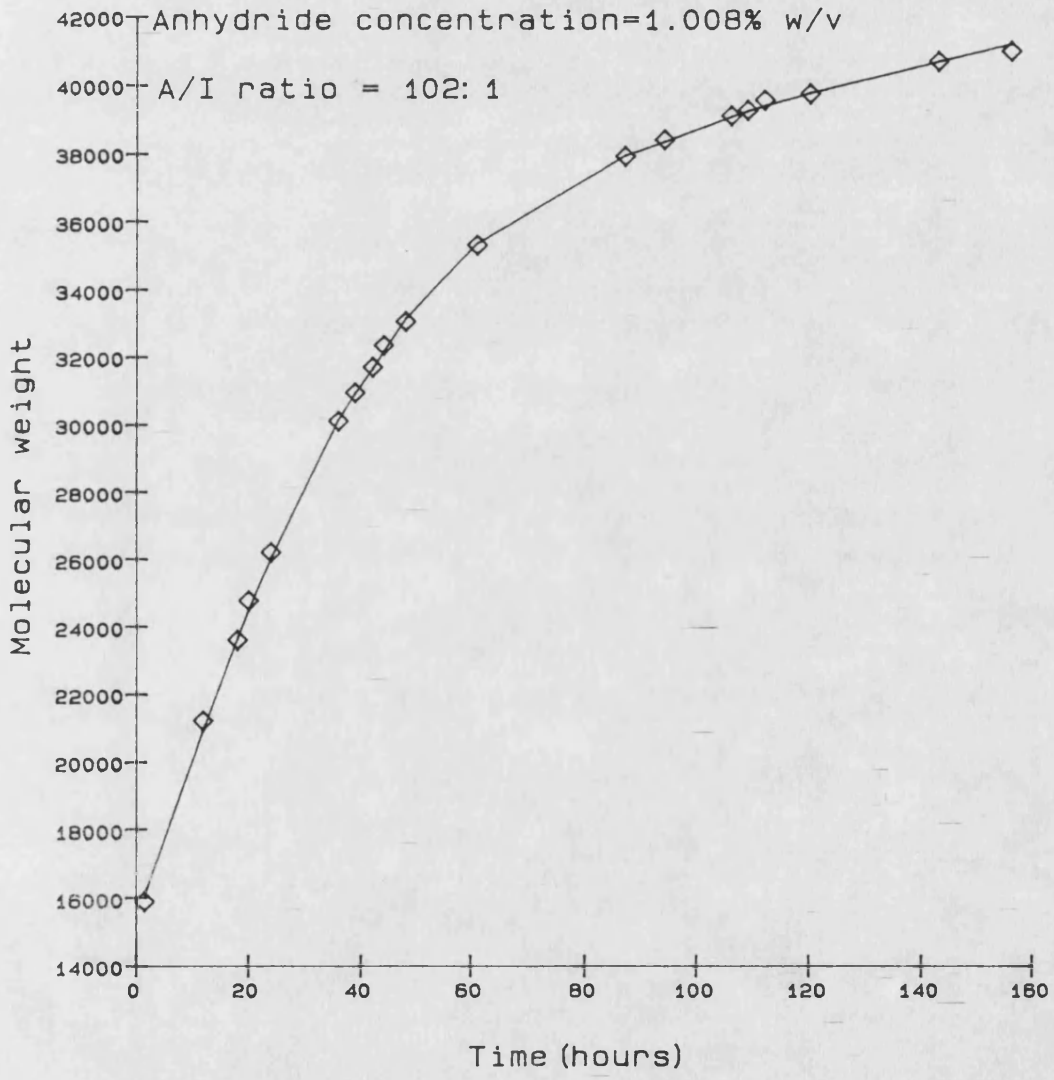
$[\eta]$ = η sp/c at infinite dilution = intrinsic viscosity

Table 4.1:
The approximate molecular weight of a protected copolymer of lysine and glutamic acid as determined by viscosity measurements in DMF (25°C)

Anhydride concentration = 1.008% w/v
Anhydride/Initiator (DEA) = 102:1

Time (Hours)	$[\eta]$ dL/g	Approx DP*	Approx MW
1.5	0.0500	66	15,900
12	0.0711	88	21,200
18	0.0819	98	23,600
20	0.0866	103	24,750
24	0.0929	109	26,200
36	0.1108	125	30,100
39	0.1146	129	30,950
42	0.1180	132	31,700
44	0.1212	135	32,350
48	0.1244	137	33,050
61	0.1351	147	35,300
87	0.1477	158	37,900
94	0.1501	160	38,400
106	0.1537	163	39,100
109	0.1543	163	39,250
112	0.1559	165	39,550
120	0.1569	165	39,750
143	0.1616	169	40,700
156	0.1630	170	41,000

Fig.4.1 The approximate molecular weight of protected lysine-glutamic acid copolymer during polymerisation as determined by viscosity measurements in DMF (DEA as initiator)



The intrinsic viscosity can be derived from one point determinations using the Simon-Cuita equation shown below (Rabek, 1980):

$$[\eta] = (2(\eta sp - \ln \eta rel)^{1/2})/c , \quad \text{usual units dL/g}$$

CLA (64.4mg; 2.10×10^{-4} m) and BGA (56.2mg; 2.14×10^{-4}m) were dissolved in DMF and 4.17×10^{-6}m of diethylamine (DEA) in DMF were added to produce a total volume of 12ml (A/I = 102:1, concentration = 1.008% w/v). A magnetic flea was added to the bowl of the viscometer to stir the solution (switched off while viscosity determination were being performed) and the tubes were sealed between determinations. The flow times were determined over a seven day period, two determinations were routinely performed within twenty minutes of each other and from these the intrinsic viscosities and apparent molecular weights were calculated (Table 4.1).

The approximate DPs (degrees of polymerisation) were determined from viscosity determinations on polycarbobenzoxylysine in DMF (Yaron and Berger, 1963). The material from this experiment was recovered by precipitation with 0.1N HCl; the precipitate was then dried over phosphorus pentoxide under vacuum and then redissolved in DMF and its viscosity determined in a cleaned viscometer $[\eta]$ = 0.1699 dl/g (DP = 176, MW = 42,350).

The results of three further experimental runs are summarised in Table 4.2.

Table 4.2 Viscometric analysis of the copolymers

Sample	BGA/CLA	A/I	Reaction time (hours)	DP	MW	Theoretical deprotected MW
COP	1	206	142	189	45,400	31,950
CPM	1	200	241	212	50,950	35,800
CP1	1	102	156	173	41,675	29,250

4.3 Amino acid analysis of poly(amino acid)s

The samples for analysis were dissolved in methanol then added to Pyrex tubes and evaporated to dryness over night under vacuum. 200ul of 6N HCl were added to the sample, the neck of the tube heated and drawn out then the liquid was frozen on dry ice/ethanol. The tube was then evacuated and the neck sealed (whilst under vacuum) followed by incubation at 105°C for 24 hours. The tube was then opened and the contents dried under vacuum overnight. The samples were reconstituted in acid and determined using standard methods; a pH gradient on an ion-exchange column (norleucine as internal standard). The samples were derivatised (post-column) with ninhydrin and then assayed colourimetrically. Standards of all 20 amino acids (50 nanomolar) were run before and after the test samples; sample concentration could thus be calculated from the areas under the curve (integrator).

Samples of two batches of copolymer (COP and CPM) were analysed using the above method. At low levels (ie. 5-10ug of polymer) both were determined

to contain 50% of each amino acid.

4.4 Synthesis of MTX-poly(amino acid) conjugates

The methods and reaction used in this experiment were essentially the same as for the HSA conjugation detailed in section 2.2.5.

20.1mg of copolymer were dissolved in 3.2ml of PBS pH7.4; 0.5ml of this solution (3.125mg = 1.25 x 10^{-7}m) were placed in a vial and the appropriate volume of MTX solution (20mg/ml), ECDI solution (42.2mg/ml) and PBS were added to yield the required reaction mix (total volume = 1.25ml). The mixes were stored at 25°C for 24 hours and then 1ml aliquots were separated on a Sephadex G15 column using PBS pH7.4 as the mobile phase, the conjugate peak being collected in 20ml volumetric flasks and analysed by UV spectrophotometry at 376nm. The MCR's were then calculated from these results (Table 4.3)

Fig.4.2 Effect of ECDI concentration on the MCR of MTX-L-copolymer conjugates (25°C, PBS pH7.4)
Molar Ratio COP: MTX: ECDI=1: 88: x

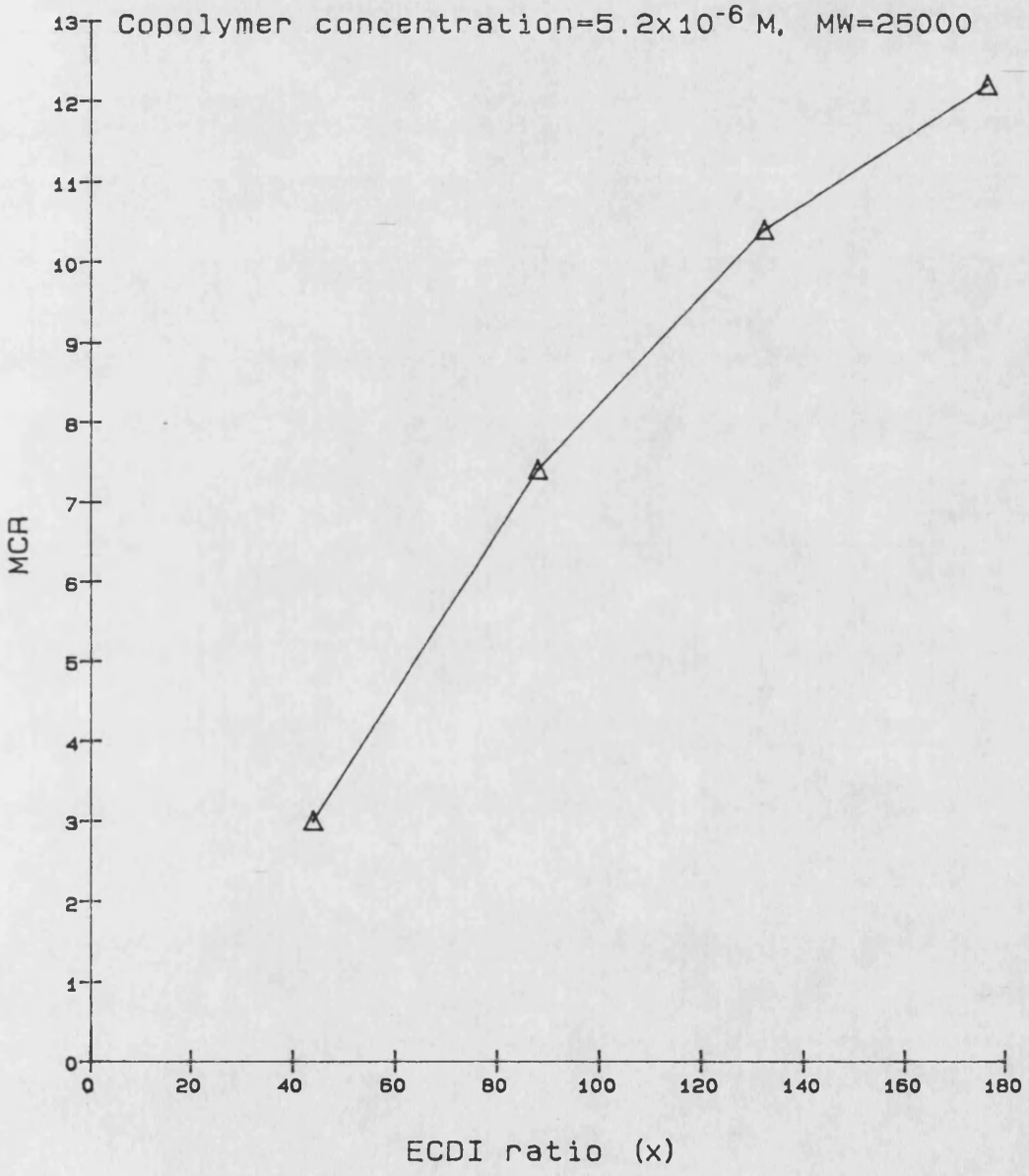


Fig.4.3 Effect of concentration of MTX and ECDI on the MCR of MTX-L-copolymer conjugates ($25^{o}C$, PBS pH7.35)
(Molar Ratio COP: MTX: ECDI=1: x: x)
Copolymer concentration=5.2×10^{-6} M, MW=25000

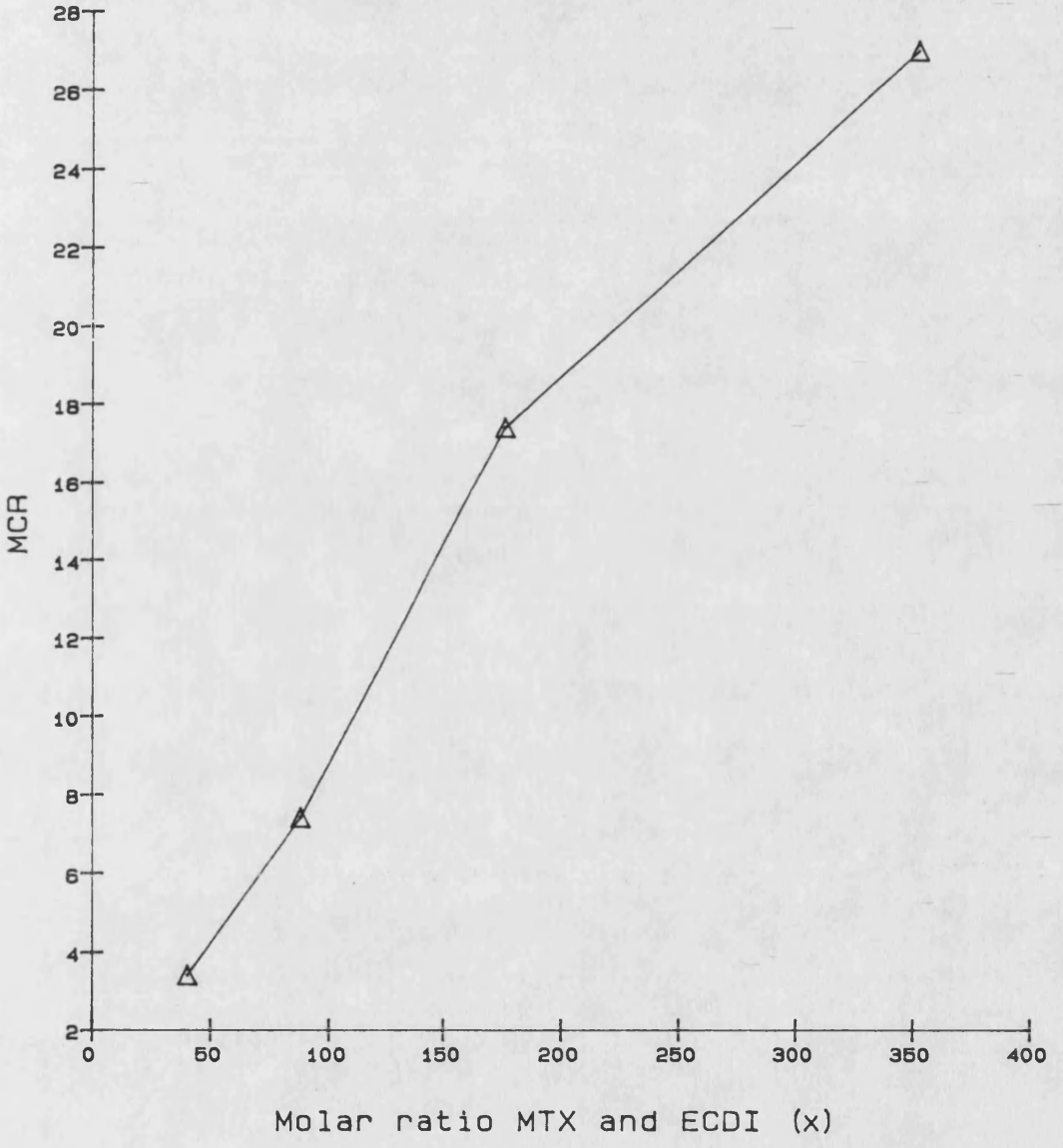


Table 4.3 Details of MTX-L-copolymer conjugates produced

Sample	Polymer : MTX : ECDI ratio	MCR
A	1 : 88 : 44	3.0
B1	1 : 88 : 88	7.4
B2	1 : 176 : 176	17.4
B3	1 : 352 : 352	27.0
C	1 : 88 : 132	10.4
D	1 : 88 : 176	12.2

4.5 The effect of trypsin on the molecular weight distribution of MTX-L-copolymer conjugates

The copolymer samples used were those produced in section 4.4. The samples were sterile filtered (0.2um Acro) and 250 ul aliquots placed in vials (0.13mg/ml). A trypsin solution was prepared in PBS pH7.4 (1.244mg/ml) and filtered; 25ul samples were then added to the copolymer solution resulting in a molar ratio of 1:1. The vials were then sealed and mixed by vortexing followed by incubation at 37°C after which the samples were frozen prior to assay on the TSK system. A sample of D-copolymer, obtained from Sigma, (at the same concentration) was prepared in a similar manner. Assay results for trypsin degradation experiments are shown in Table 4.4

A further experiment was performed using MTX-copolymer sample B1 (see section 4.4). A 500ul sample of B1 solution (in PBS pH7.4) was placed in

Table 4.4:The molecular weight distributions of MTX-L-copolymers following incubation with trypsin in buffer pH 7.4 at 37°C for 24 hours

Trypsin:copolymer molar ratio = 1:1
Copolymer concentration = 0.13 mg/ml

	Molecular Weight x 10^3								
Sample	70-65	55-50	35-30	30-25	15	8	3.5	2	<1.5
A	-	-	-	9.5	8.7	11.8	19.4	4.8	46.0
B1	-	-	-	10.3	10.8	15.5	21.7	4.5	37.3
B2			75.9	-	-	8.6	9.6	-	5.9
B3	97.6	-	-	-	-	-	-	-	-
C				28.5	10.2	16.2	21.4	-	23.9
D	-	-	56.3	-	-	11.6	15.5	-	16.6
D-copolymer	-	100.0	-	-	-	-	-	-	-

Results expressed as % of total area

a vial, a 50ul aliquot of trypsin solution (0.124mg/ml) was added and the solutions mixed by vortexing. The resulting molar ratio of copolymer : trypsin was 10 : 1.

100ul portions were dispensed into 500ul vials and stored at 37°C prior to assay. Samples were assayed on the TSK column at 0, 2, 6, 16 and 24 hours. A blank solution was prepared by replacing the trypsin solution with PBS buffer pH7.4; these samples were also stored at 37°C and assayed at 0,2,6 and 24 hours. Data are shown in Table A8.4.1 and plotted in Figure 4.4. The blank samples exhibited little alteration in peak shape over the period of analysis with the bulk of the peak 94-99% having a molecular weight around 46,000. The molecular weight distribution following 24 hours at 37°C is displayed graphically in Figure 4.5. The overall molecular weight distributions were analysed using the molecular weight analysis programme (Table 4.5)

Fig.4.4 Effect of trypsin on the molecular weight distribution of MTX-L-copolymer conjugates ($37^{o}C$, PBS pH7.4)

Copolymer conc.=4.73×10^{-6} M, MW=25000, Try: COP=1: 10

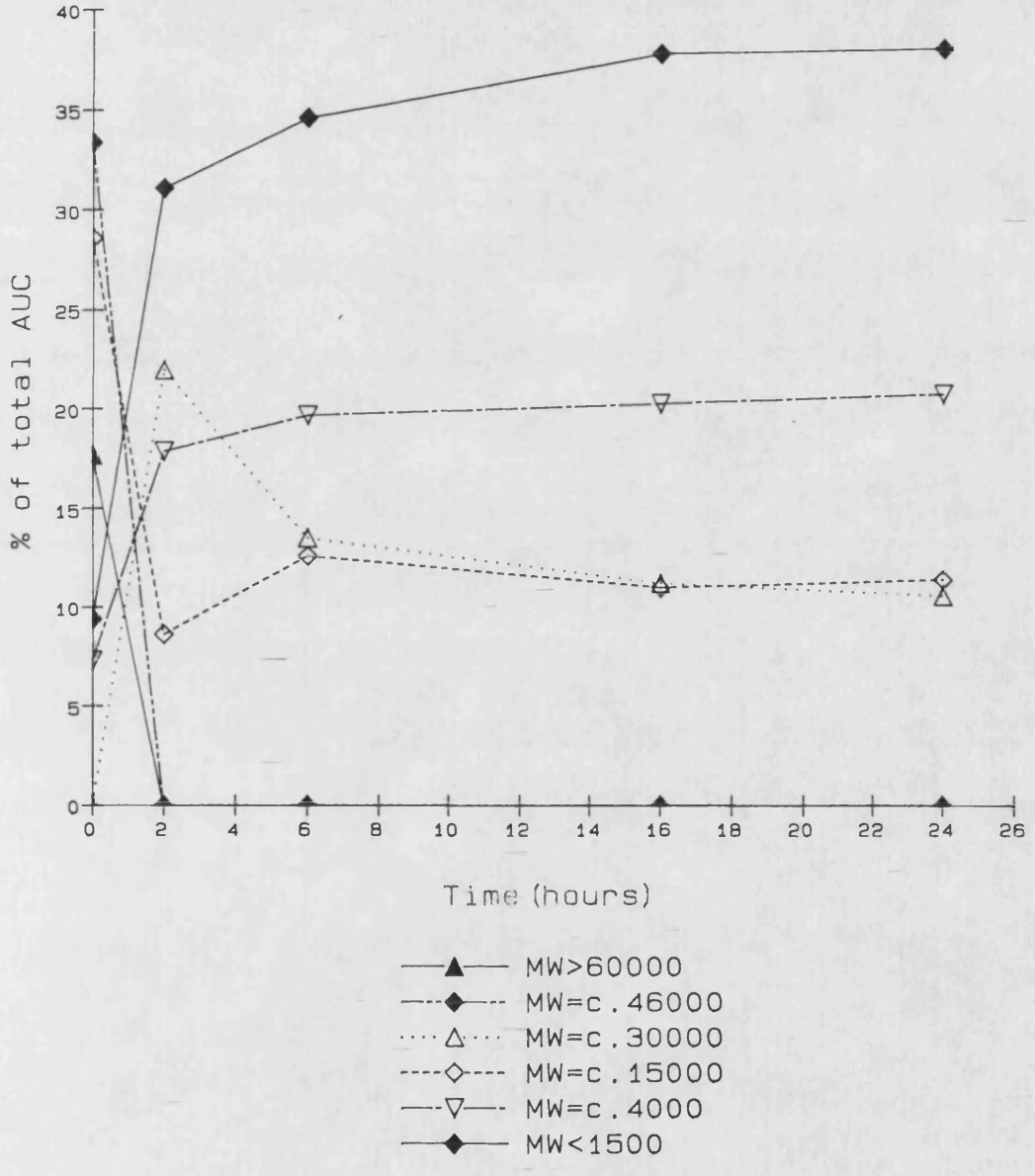


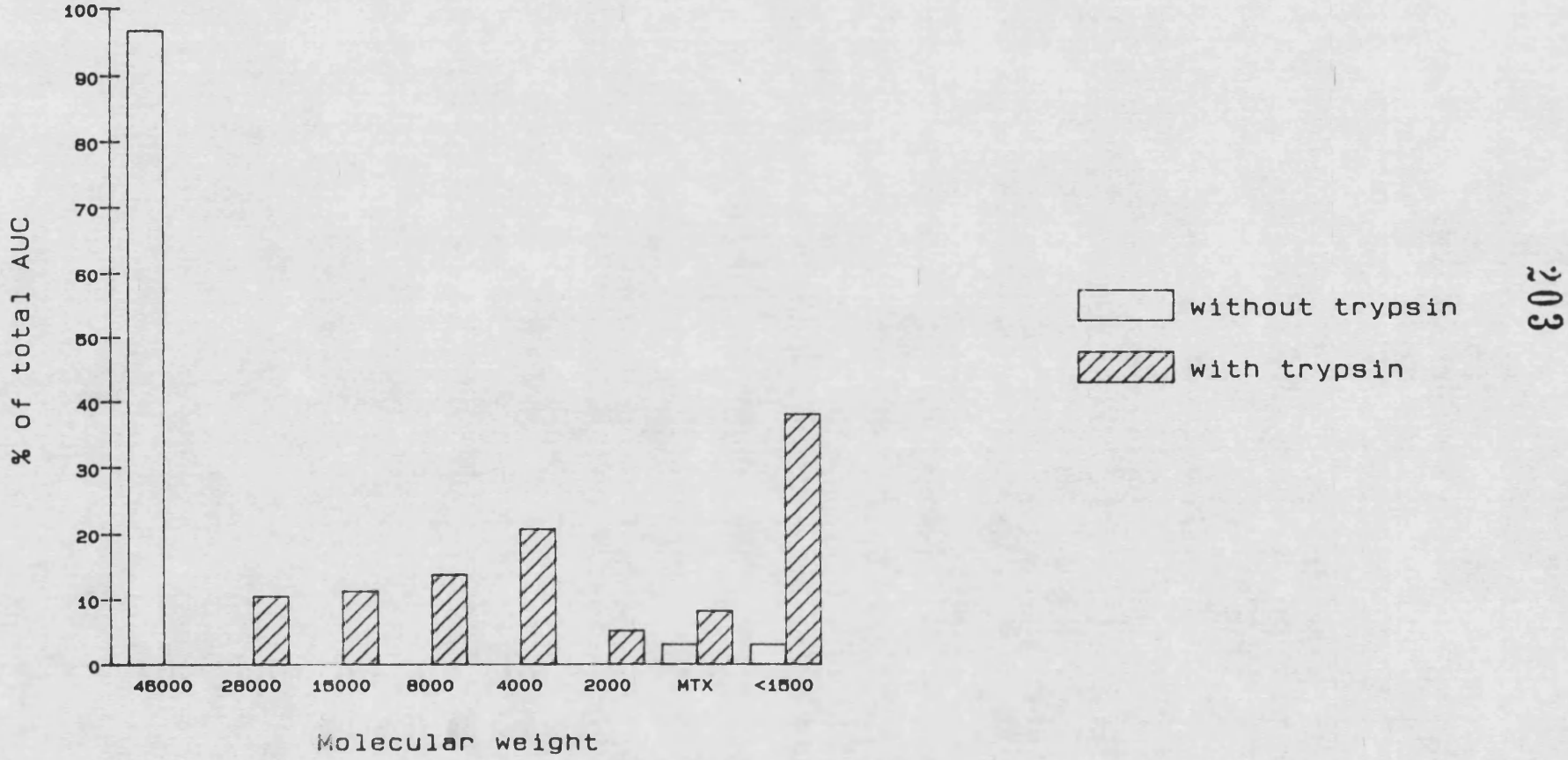
Table 4.5:
The overall molecular weight distributions of MTX-L-copolymer conjugates using a fixed baseline, in the presence and absence of trypsin in buffer (pH 7.4) at 37°C

Sample	AUC	Mw x 10^3	Mw/Mn
BLA-0H	12.2	42.6	5.8
BLA-2H	12.5	39.6	4.6
BLA-6H	16.5	37.2	6.6
BLA-24H	23.1	35.5	8.4
TRY-0H	37.8	36.4	8.8
TRY-2H	41.9	9.6	8.2
TRY-6H	45.8	8.0	8.7
TRY-16H	45.2	7.4	7.3
TRY-24H	45.0	6.7	7.8

BLA = Blank
TRY = Trypsinised sample
(molar ratio = Try:copolymer = 1:10)
-xH = incubation time in hours
Copolymer Concentration = 4.73 x 10^{-6}M
MW = 25,000

Fig.4.5 Effect of trypsin on the molecular weight distribution of MTX-L-copolymer (37°C, 24 hours) (Try: COP=1: 10)

COP concentration=4.73×10^{-6} M, MW=25000 (PBS pH7.4)



4.6 The preparation and stability of MTX-D-copolymer conjugates

Conjugates were prepared using a copolymer obtained from Sigma Chemical Co Ltd comprising D-glutamic acid and D-lysine (60:40, MW 24,000); the MTX being linked to the copolymer using the ECDI mediated reaction.

The reaction mixture comprised D-copolymer (19.7mg), ECDI solution (25mg/ml) and MTX solution (20mg/ml) in a total volume of 2.0ml; the molar ratio was 1:64:43. The medium was PBS pH7.4 however the pH of the reaction mixture was measured as 6.0. The reaction was allowed to proceed at 25°C for 26 hours before separation on a Sephadex G15 column. 1.04ml were applied to the column and collected in 46ml, diluted to 100ml with PBS prior to filtration (0.2um) and assay by UV spectrophotometry; the MCR was found to be 8.73.

The conjugate solution was assayed using the Superose 12 system (300nm) by repeated sampling from a 50ml vial at 37°C. The chromatogram showed the main peak (conjugate) eluting at 19 minutes, which corresponded to a molecular weight in excess of 200,000, and a small amount of free MTX (initially 0.33%) eluting after 50 minutes. The level of free MTX had increased to 0.5% after 12 hours and 0.75% after 89 hours at 37°C. There was no apparent alteration in the molecular weight distribution with the retention time being constant at 19 minutes and the peak width at half height remaining constant at 5.75 mm.

A further experiment was performed using an identical reaction mix. The conjugate solution was incubated at 37°C and then assayed on the TSK system at various time points. The chromatograms comprised one broad peak

Table 4.6:
The effect of storage on the molecular weight distribution of MTX-D-copolymer conjugates (PBS, pH 7.4, 37°C)

Copolymer Concentration = 0.512 mg/ml
MW = 24,000

Storage Time at 37°C	Molecular Weight x 10^3			Overall Molecular Weight x 10^3 Mw	Mw/Mn	% Free MTX
	100-90	7-6	3-2.5			
Initial	-	-	-	-	-	0.00
1 hr 20 min	83.6	6.5	-	-	10.6	0.30
2 hr 40 min	91.4	-	3.0	80.8	10.6	0.33
3 hr 55 min	85.1	6.5	2.5	85.5	10.2	0.37
5 hr 10 min	85.6	6.9	2.3	87.3	9.7	0.48
6 hr 25 min	85.7	6.9	2.1	89.2	9.5	0.59
7 hr 40 min	85.9	7.0	2.6	91.8	9.1	-
8 hr 55 min	85.5	7.1	2.5	91.8	9.8	-
26 hr 45 min	84.4	7.0	2.9	93.7	11.1	1.03
38 hr	-	-	-	-	-	1.10
47 hr 18 min	88.2	4.6	4.1	-	-	1.18
50 hr 39 min	86.5	6.0	2.6	-	-	1.14
51 hr 54 min	-	-	-	-	-	1.18
53 hr 9 min	-	-	-	-	-	1.18

with a slight shoulder at a MW of approximately 7000 and a small peak at 3,000. The chromatogram was also analysed for overall molecular weight distribution. The level of free MTX was determined from peak height measurements. Data is shown in Table 4.6

4.7 The effect of tritosomes on the molecular weight distribution and release of free MTX from MTX-D-copolymer conjugates

The production of the conjugate has been outlined in Section 4.6.

A sample of the conjugate was thawed and filtered (0.45um), the pH of the sample was adjusted to pH6 using citric acid and the following mixtures were prepared:

	Tritosomes test sample vol(ml)	Blank sample vol(ml)
MTX-copolymer (0.102mg/ml)	3.33	3.33
10% surfactant	0.1	0.1
EDTA solution (17.4mM)	0.288	0.288
Reduced Glutathione (86.4mM)	0.288	0.288
H_2O	0.034	0.034
Tritosomes	1.0	-
Sucrose 20%	-	1.0
Total volume	5.04ml	5.04ml

Table 4.7:
The effect of tritsomes on the molecular weight distribution of MTX-D-Copolymer conjugates (buffer pH 6, 37°C)

Copolyer Concentration = 0.102 mg/ml
MW = 24,000

Sample/Storage time (h) at 37°C	Molecular Weight x 10^3 100-90*	Overall Mw x 10^3	Mw/Mn	% Free MTX
Initial - T	97.5	93.9	3.1	0.30
1H - T	96.6	100.2	3.1	0.61
2H - T	96.4	89.7	3.0	0.51
4H - T	96.7	95.2	3.1	0.53
16H - T	96.1	97.4	2.9	0.68
25H - T	96.2	93.6	3.0	0.63
49H - T	95.4	85.4	2.7	0.85
16H - B	98.9	103.4	2.1	0.53
25H - B	97.8	112.3	2.9	1.04
49H - B	98.9	98.4	2.7	0.61

* Results expressed as % total area under curve

T = with tritosomes
B = Blank

The samples were then placed in vials and incubated at 37°C prior to assay on the Superose 12 system. The chromatograms showed a broad main peak with a second peak after about 34 minutes which was due to the enzyme/buffer system. A further blank containing enzyme etc. but no copolymer was run and the chromatogram subtracted from the copolymer and enzyme samples; the overall molecular weight was then calculated from these chromatograms. The level of free MTX was also calculated from peak heights by comparison with an MTX standard. Data is shown in Table 4.7.

4.8 The effect of tritosomes on the molecular weight distribution of MTX-L-copolymer conjugates

Conjugate was produced as for the D-copolymer (Section 4.6) yielding a final copolymer concentration of 0.2mg/ml with a molecular weight distribution of approximately 100,000. The conjugate was fractionated in buffer at pH 5 and the digestion mixtures produced according to that described in Section 4.7. Three solutions were produced ie. without tritosomes, with tritosomes but without reduced GSH and with tritosomes and reduced GSH. These were incubated at 37°C for up to 7 days prior to analysis on the Superose system. The results are presented in Table 4.8 as the areas under the curve for the main conjugate peak and for low molecular weight material (with retention greater or equal to that of MTX).

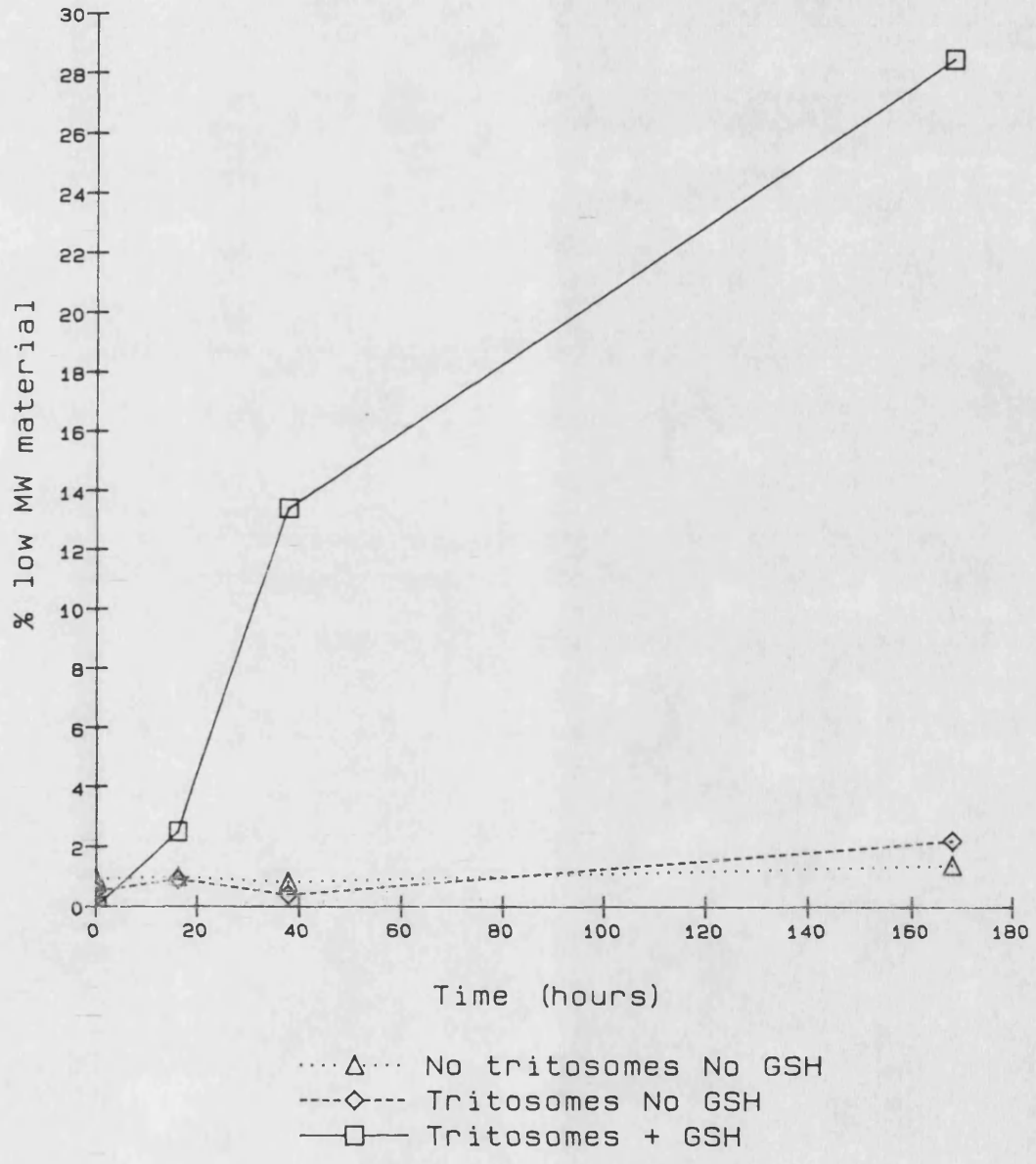
Table 4.8: The tritosomal degradation of MTX-L-Copolymer conjugates in buffer (pH 5, 37°C)

	No tritosomes		Tritosomes - No GSH		Tritosomes + GSH	
Peak No	1	4	1	4	1	4
Incubation Time	MTX-COP	low MW MTX derivs.	MTX-COP	low MW MTX derivs.	MTX-COP	low MW MTX derivs.
Initial	92.0 (103)	0.9	93.4 (95.7)	0.5	93.7 (90.6)	0
16h	93.4 (114)	1.0	91.3 (89.1)	0.9	71.8 (46.7)	2.5
38h	90.0 (99.6)	0.8	96.7 (81.5)	0.4	49.6 (±6.6) (30.9)	13.4 (±0.2)
7 Days	93.8 (104)	1.4	79.5 (±3.6) (84.8)	2.2 (±0.1)	20.6 (±6.9) (35.9)	28.5 (±0.2)

Results expressed as % of total area

MW = molecular weight x 1000 (in parentheses)
GSH = reduced glutathione

Fig.4.6 The tritosomal degradation of MTX-L-Copolymer conjugates in the presence and absence of reduced glutathione (in buffer pH5, 37°C)



4.9 Discussion

The L-copolymersof lysine and glutamic acid were prepared from NCA's of the parent amino acids. This was achieved by protection of both amino groups in the case of lysine by forming the dicarbobenzoxy derivative; the anhydride being formed via the acyl chloride using phosphorus pentachloride. The NCA of glutamic acid was produced by forming the benzyl derivative of the γ-carboxylic acid and the carbobenzoxy derivative of the amine group; the anhydride once again being formed by using PCl_5. The composition of these intermediate compounds was confirmed by ^1H NMR and ^{13}C NMR as well as melting point determinations.

Copolymerisation of the NCA's was performed in DMF with initiation using a secondary amine; the structure of these polymers could not be determined by NMR due to the breadth of the peaks however the presence of benzylic protons was obvious (7.3 ppm). The copolymer was deprotected using hydrogen bromide in glacial acetic acid thus forming the hydrobromide salt of the lysine. The absence of benzyl groups were confirmed by UV and NMR (as well as the increase in aqueous solubility). The polymerisation reaction was followed by viscometry to determine the optimum time to allow the reaction to proceed before termination; this showed that the reaction slowed after about 150 hours and that any time in excess of this should yield a polymer of suitable molecular weight (ie. approximately 25,000 Daltons).

The intrinsic viscosity was determined using a one point determination of

the viscosity. To determine the viscosity at a series of concentrations would have proved impractical at each time point although this would have yielded a more accurate value upon termination. The accurate determination of the intrinsic viscosity was considered inappropriate since the molecular weight/degree of polymerisation was determined from published values for poly-L-lysine and would thus only give an estimate of molecular weight for the copolymer. The viscosity experiment did prove useful since it showed that the reaction resulted in an increase in viscosity (and thus molecular weight) and gave an indication of the required reaction time.

The molecular weight of the deprotected copolymer was determined from the degree of polymerisation for the protected copolymer. This assumes (as has previously been demonstrated with polylysine) that treatment with HBr does not affect the integrity of the polymer backbone. The copolymers produced had theoretical molecular weights of 30-35,000; analysis on the TSK column showed the weight av. MW to be of the order of 37,600.

Amino acid analysis confirmed that the only amino acids present in copolymer samples were glutamic acid and lysine at the input levels of 50mole% of each.

It has been reported previously that this method of preparation does not alter the configuration of the amino acids such that the resulting copolymer will also contain residues with the L-configuration. The configuration of the input amino acids and protected derivatives was confirmed by optical rotation measurements (compared to literature values); it was further confirmed by the ability of trypsin to degrade

samples conjugated with MTX and the inability of the enzyme to degrade a MTX conjugated derivative of the D-copolymer.

The conjugation of MTX to copolymer was achieved using the same carbodiimide-mediated reaction employed when HSA was used as the carrier macromolecule. The MCR was calculated in a similar manner using the quoted molecular weight of 24,000 for the D-copolymer and a nominal value of 25,000 for the L-copolymer. The results showed an increasing MCR with increasing level of ECDI (for a copolymer:MTX molar ratio of 1:88) reaction efficiency increasing from 3 to 15% (ECDI = 44 to 176). The MCR also increased with increasing molar ratio of MTX and ECDI (at a fixed ratio of 1:1) reaction efficiency peaking at 10% at 176. The use of ECDI in addition to linking MTX to the copolymer also brought about an increase in the molecular weights of the conjugates the effect being more noticeable at the higher levels of ECDI. This was probably as a result of amide bond formation between the carboxylic acid residues of the glutamic acid and the amine of lysine mediated by the carbodiimide causing further polymerisation and/or cross linking with an associated increase in molecular weight.

The addition of equimolar proportions of trypsin to the solutions and incubation at 37°C for 24 hours in some cases brought a major change in the molecular weight distribution and in other cases had no effect at all. The chromatograms were all analysed without subtraction since the alteration in peak area due to the trypsin was minimal.

As expected the MTX-D-copolymer sample was not degraded at all by trypsin due to the fact that it was not composed of naturally occurring amino

acids. The L-copolymers, with the exception of B3 (see section 4.4), all exhibited some degree of breakdown although the amount of material below MW 1,500 was found to decrease with increasing MCR. This effect also corresponded to increasing levels of ECDI thus the enzymatic resistance may thus be explained by an increase in cross linking. However an examination of incubated samples of B2 and D reveals that more extensive degradation of the latter sample occurred, which had the lower MCR, although the levels of ECDI in the reaction mixture were equivalent. This implies that the tryptic hydrolysis of the lysine peptide bond was impeded by the presence of conjugated MTX, which may be explained since the lysine amino group will not be protonated as it is derivatised which may prevent enzymatic hydrolysis. The MW bands that resulted from the degradation of the conjugates are fairly consistent although B2 and D exhibited a reduced number of peaks, with the main peak being fairly broad. A repeat of this experiment employed conjugate B1 only and trypsin at the lower level of 10:1 molar ratio (copolymer : trypsin). The initial predominant peak was at 46,000 although other peaks existed at lower molecular weights, this was thought to be due to the time between addition of the enzyme and assay. The high molecular weight peak rapidly disappeared and was replaced by a peak at 30,000 which subsequently diminished (Figure 4.4). The peaks appearing in the other molecular weight bands remained at a fairly constant level after 2 hours. The amount of low molecular weight (below 1,500) material rose rapidly to achieve a level of 38% after 24 hours (Figure 4.5), the observed distribution was in fact not significantly different from that with the higher level of trypsin.

The overall molecular weight distribution of the blank samples showed a slight decrease from 43,000 to 36,000 and a slight change in the

polydispersity index (Table 4.5). The trypsinised sample on the other hand exhibited a large drop from 36,000 to 7,000 over the same 24 hour incubation period with no change in the polydispersity index. The latter result was caused by the rapid change from material at high molecular weight to that at low molecular weight. The area contribution due to trypsin in these samples was relatively small and not subtracted from the samples. However analysis of the areas under the curve revealed a large discrepancy between the trypsinised samples and the controls (about 3 fold). As yet this phenomenon cannot be explained.

The results of the tritosomal degradation experiment (Section 4.8, Figure 4.6) demonstrated that the conjugate was broken down by the enzymes and that as previously shown for HSA conjugates this was dependent on the presence of reduced GSH. The data displayed in Table 4.8 only shows the high molecular weight conjugate peak and low molecular weight material being retained for times equal to or in excess of MTX. The main intermediate peak was observed at 3-5kD but was subject to variability due to interference in the chromatogram with the tritosomal solution.

CHAPTER FIVE: GENERAL DISCUSSION AND CONCLUSIONS

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The use of macromolecules to alter drug distribution, uptake and selectivity has great potential in cancer chemotherapy. This study has investigated the linking of a cytotoxic agent to soluble carrier systems as well as their solution stability and enzymatic degradability in order to help to explain their modes of action at a cellular level. The macromolecular carriers used were the naturally occurring human serum albumin and synthetic polypeptides.

HSA was used as a carrier for investigation since it possesses a variety of functional groups for the attachment of drug molecules/targeting agents and would be able to remain soluble. The molecule itself is large enough to escape glomerular filtration and small enough to be extravasated which are important features of macromolecular carriers. Serum albumin is known to be taken up by pinocytosis and its uptake increased by modification eg. formaldehyde treatment (Moore et al, 1977). HSA has also been shown to be degraded by lysosomal enzymes which is a prerequisite for prodrug action. The modification of HSA however may lead to increased immunogenicity.

The linking of MTX to HSA is likely to occur via pendant amino groups since both the carbodiimide and NHS ester mediated reactions are via nucleophilic attack. The amino acid residues that may be involved in this are histidine, arginine and lysine. Histidine is unlikely to react due to its ring structure; arginine has a high pK_a (>12) and would be charged under the reaction conditions, the lone pair would also be delocalised into the double bond. Lysine (ϵ-NH_2) is thus the only residue that could form an amide bond with the carboxylic acid residue of MTX, evidence for

this is the ease of conjugation of MTX to poly-L-lysine and copoly-L-lysine-L-glutamic acid. The ECDI reaction may also give rise to ester links eg with hydroxyl groups present on serine or threonine residues which would account for the increased release of ECDI-linked MTX over that released from the NHS-mediated product which will only react with amine residues. Further evidence for the ester formation is supplied by the fact that the release is base catalysed and that MTX can actually be linked at a low level to polyvinylalcohol which contains only hydroxyl groups. The similar levels of release observed from NHS linked MTX-HSA, refractionated ECDI linked MTX-HSA and ECDI linked MTX-copolymer indicates that the amide bond itself is hydrolysed, since the free MTX is not derived from protein bound material. A 'stable' carbodiimide produced conjugate of MTX-HSA was produced by briefly exposing it to elevated pH prior to fractionation. A low level of MTX was also observed following incubation at pH 11 once again pointing to amide hydrolysis since the elevated pH should rapidly hydrolyse any ester bonds. The conjugation reaction in this case was performed in water however this gave rise to an increased level of protein cross linking (ie higher molecular weight derivatives). It would thus be preferable to perform the conjugation reaction in a buffer solution prior to increasing the pH of the system.

The release of free drug by simple hydrolysis extracellularly from a conjugate is undesirable as in cell culture it may lead to distorted results, since part of the cytotoxicity would be due to free drug as opposed to lysosomally degraded endocytosed conjugate (Chu and Whiteley, 1979).

The results presented in this paper show that 10^{-4}M MTX-BSA was as effective in vitro against L1210 cells as 10^{-6}M MTX following 1 hour

exposure at 37°C. The conjugation reaction time (ECDI mediated) was only 4 hours followed by dialysis/lyophilisation; the results presented in this thesis suggest that 1% free MTX could be released from the conjugate following 1 hour at 37°C. The cytotoxicity reported by Chu & Whiteley (1979) is thus probably due to MTX hydrolysed from the conjugate resulting in 10^{-6}M free MTX from 10^{-4}M MTX-BSA

The tissue culture experiments of Halbert (1987) demonstrate a similar relationship in that a higher concentration of bound drug was required to achieve the same level of inhibition as free drug. It is likely that the majority of this activity could once again be accounted for by the hydrolytic release of MTX as opposed to the cytotoxicity being caused solely via an intracellularly mediated mechanism.

The release of free drug could also affect the selectivity of conjugates linked to antibodies when tested in cell culture experiments since hydrolysis of the drug would reduce the selectivity ie a targeted conjugate may be more specific than the data suggest. The work of Garnett et al (1983) demonstrates that there is a 1000 fold difference between the activity of free drug and MTX-HSA; the activity of the conjugate could once again be caused solely by the release of free MTX.

The simple extracellular hydrolysis of drug-macromolecular conjugates can have significant effects on the results of cell culture experiments. It is essential to produce a system that is completely stable in the extracellular medium or at lease to quantify the release in order to explain the results of in vitro cell experiments.

The linkage of MTX to an amine could be via either the α or γ carboxylic acid residue although it is likely that the former may be more cytotoxic as has been demonstrated by Rosowsky et al (1984). This group found that MTX which is linked via the γ carboxyl moiety to PLL was more active in terms of DHFR inhibition and in cytotoxicity than the corresponding α carboxyl derivative.

The method of choice for linking MTX to carrier is debatable since both ECDI and NHS have advantages and disadvantages: the former can be used in a one step aqueous reaction but can also cause side reactions (ester formation and cross-linking) and is relatively inefficient. The NHS reaction is more efficient and results in a more stable product however it is a two step reaction and the presence of DMF may result in insolubility of the carrier and a reduced maximum molar conjugation ratio. It may be possible to improve the efficiency of the ECDI reaction by the addition of N-hydroxysuccinimide to the reaction mixture (which may also result in reduced cross-linking and other side reactions). This would be an interesting line of future research. Cross-linking or polymerisation would lead to an increase in molecular weight with consequent effects on the biodistribution caused by changes in the ability of the molecule to leave the vasculature (Halbert et al, 1989).

An improvement of the NHS method would be to form a water soluble salt of the ester eg. the sodium sulphonate salt of NHS-MTX.

HSA has limited potential as a carrier for MTX since at high MCR's the solubility at low pH is compromised. This is important since it is intended as a lysosomotropic agent and the insolubility could result in

reduced degradability. The presence of the dimer may also cause problems in biodistribution studies since the higher molecular weight dimer would have a different distribution to the monomer. The potential restriction on the MCR coupled with the limited toxicity of MTX would require a large number of conjugate molecules to achieve a cytotoxic concentration intracellularly, if these were linked to a vectoring agent then the molecular weight may prove to be too great to achieve extravasation.

The use of a homopolymer of lysine as a carrier system has the potential advantage of maximising the number of sites for the formation of amide bonds (ie linking MTX) whilst keeping the molecular weight to a minimum and maintaining solubility. The use of poly-L-lysine is however restricted due to its inherent cytotoxicity since its cationic nature causes it to adsorb onto cells non selectively. The net charge of the polypeptide can be reduced by the incorporation of an anionic residue, eg. glutamic acid, however polymerisation of equimolar proportions of the NCA's of lysine and glutamic acid will not produce a sequential copolymer due to the reaction mechanism. The result is close to a random copolymer, however the different rates of reaction (Shalitin and Katchalski, 1959) of NCA polymerisation are expected to result in regions of highly positive and negative charge density which may still result in non-selective binding. The loading of MTX onto the copolymer may once again be limited at lower pH due to solubility problems; this may be improved in future work by incorporation of amino acids bearing free hydroxyl groups eg serine.

The link between MTX and lysine may once again be effected using ECDI however this can also bring about an increase in molecular weight due to

cross linking of polymer chains. This would also cause problems of esterification were serine to be included. The use of a water soluble NHS derivative is thus desirable in future work since the presence of DMF caused solubility problems (of the copolymer) which precluded the use of the NHS mediated reaction. The MTX-copolymer conjugates were found to be more stable in buffer solutions than MTX-HSA however a low level of free MTX was still released.

The release of drug from the conjugates in this study was determined in isotonic buffer, it may be that the release profile would be different in plasma due to the presence of low levels of enzymes and also other proteins (which would bind any free drug). This analysis, while possible to monitor using UV (at 370nm), would be more easily facilitated by employing radio labelled drug. Thus the use of a flow-through radiodetector coupled to the FPLC system would be a powerful tool for future work.

The enzymatic degradation of the conjugates was initially tested with trypsin as a model enzyme to check and develop the FPLC/molecular weight analysis programmes (this also has some application to the in vivo situation since acrosin , present in lysosomal enzymes, has a similar specificity to trypsin). The incubation of both conjugate systems with trypsin (at pH7.4) brought about a reduction in molecular weight but MTX was not released in the free form. The digestion of MTX-HSA with tritosomes brought about a dramatic change in the molecular weight with a large proportion of low MW material which is due to the presence of enzymes with different specificities. However thiol dependent proteases clearly have the most important role to play in the digestion process.

The need for the MTX-L-polypeptide conjugate has been demonstrated since MTX-poly-D-lysine was found to be inactive in cell culture experiments (Shen and Ryser, 1979); its lack of degradability has been demonstrated in this study when incubated with trypsin and tritosomes. In contrast MTX-poly-L-lysine conjugates were broken down to low molecular weight derivatives by lysosomal enzymes or trypsin.

This study has shown that polypeptide-based macromolecular prodrugs of MTX are lysosomotropic agents they are likely to be processed within the cell by lysosomal enzymes and that the enzymes likely to be involved are pH dependent, thiol proteases.

It is unlikely that MTX needs to be released in the free form to bind with DHFR since the polyglutamated derivative is active and retained in the cell longer (Jolivet et al, 1982). The pteridine moiety actually inserts into the cleft of the enzyme with the glutamate residue, particularly the γ carboxyl, being less involved in the binding (Champness et al, 1986). Thus oligopeptides of MTX may still be active and it has been shown that macromolecule-MTX conjugates still retain the ability to bind DHFR (Shen and Ryser, 1981a). However, free MTX is likely to be driven out of the lysosome due to the pH gradient (low pH leading to more unionised drug and increased lypophilicity). It is uncertain whether the derivatives would be able to leave the lysosome, since it has been reported that only molecules of molecular weight less than 200 Daltons can permeate the membrane (Forster and Lloyd, 1988), although it seems likely that they do since the conjugates are active in cell culture. This could possibly be explained by exocytosis followed by active uptake via the MTX active transport system however this seems an unlikely mechanism (Garnett et al, 1984). The nature of the low molecular weight fragments following tritosomal degradation should now be further investigated by ion exchange

chromatography and isoelectric focussing. The 'low MW material' could conceivably be large fragments with a high degree of column interaction (eg if they contained a large number of hydrophobic groups) or low MW derivatives due to the random distribution of residues in the macromolecule. Future work should be aimed at the isolation of degradation products and the assessment of their ability to inhibit DHFR activity.

The use of MTX itself may be restricted by its low toxicity necessitating high intracellular levels for cytotoxic effect. However the fact that MTX derivatives may be more active than free drug mean that it may not require a spacer to be active. An advantage of MTX is that normal, non-target cells may be rescued using leucovorin.

In order to transform the aforementioned passive carrier systems into site specific delivery system a vectoring moiety needs to be added, eg an antibody, to improve the selectivity. The drug delivery system needs to be small enough to be extravasated in order to access the desired site whilst retaining selectivity and having sufficient drug loading to be active intracellularly following degradation. A further constraint on the molecular size is that it should not be too large enough so that it may escape uptake by the reticuloendothelial system. The preferred system would be the drug (possibly more than one type with different modes of action) linked to a polypeptide with the F_{ab} portion (or better still the active site) of a monoclonal antibody as the site directing agent. It is envisaged that such systems would be used primarily to mop up metastases following excision of the tumour mass, consequently it is essential that the system can leave the circulation in order to attach to the cells and in internalised.

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APPENDIX A1: SUMMARY OF MATERIALS USED

Appendix A1 Materials

The following materials were used:

human serum albumin

Cohn Fraction V, crystallised and lyophilised

obtained from Sigma (catalogue number A9511)

poly(D-glu-D-lys.HBr)

60 mole% glutamic acid, 40 mole% lysine, DP=151, MW=24,000

obtained from Sigma (catalogue number P7568)

methotrexate

disodium salt, lyophilised

donated by Cyanamid (GB) Ltd.

trypsin

from bovine pancreas, dialysed and lyophilised

obtained from Sigma (catalogue number T8253)

All other materials and reagents were analytical grade and were used as received.

APPENDIX A2: CALIBRATION AND CHARACTERISATION OF FPLC COLUMNS

Appendix A2 Calibration of FPLC columns

A2.1 Calibration and characterisation of the Superose 12 column

A2.1.1 Molecular weight calibration

The column (Superose 12 HR 10/30) was supplied pre-packed by Pharmacia and had a claimed separation range of 1000 - 100,000 Daltons. Calibration was performed using Pharmacia protein standards. The conditions for a typical calibration run were as follows:

Flow rate:	0.48ml/min
Mobile phase:	Phosphate buffered saline pH7.4
Column Temp:	25°C
Sample preparation:	protein conc. 5mg/ml, blue dextran 1mg/ml in mobile phase (filtered 0.2um)
Sample volume:	200ul
Detection:	280nm
Column length:	28.5ml (Vt = 22.4ml, Vo = 6.4ml)

Typical calibration data are shown in Table A2.1. Regression analysis of Kav against log MW yielded the following, indicating a good linear fit:

Slope = -0.245 ± 0.014 ($p = 0.05$)

Intercept = 1.521 ± 0.062 ($p = 0.05$)

R^2 = 99.9% r = 0.9995

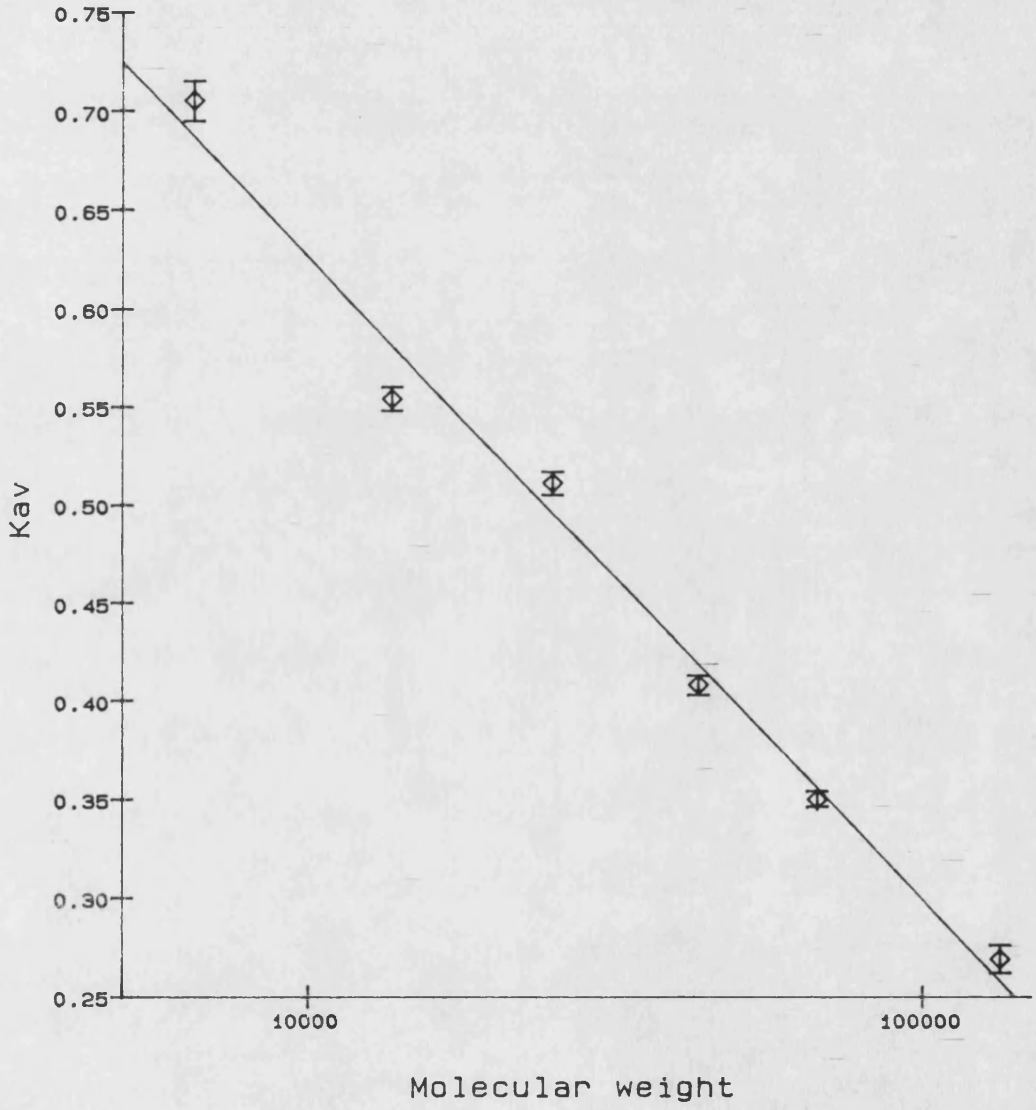
Table A2.1:
Molecular weight calibration of the Superose 12 column using protein standards (PBS, pH 7.4, 25°C, 0.48 ml/min)

Sample	Molecular weight	log MW	Elution volume Ve/ml	Kav
Bovine serum albumin	67,000	4.826	11.8	0.338
Ovalbumin	43,000	4.634	12.6	0.388
Chymotrypsinogen A	25,000	4.398	13.6	0.450
Ribonuclease	13,700	4.137	14.5	0.506
Aprotinin	6,500	3.813	15.8	0.588

Table A2.2:
Molecular weight calibration of the Superose 12 column using protein standards (including HSA-dimer) (PBS, pH 7.4, 25°C, 0.48 ml/min)

Sample	Molecular Weight	log MW	Mean Kav (sd) [n]
1. HSA-dimer	134,000	5.127	0.269 (±0.007) [5]
2. HSA-monomer	67,000	4.826	0.350 (±0.004) [6]
3. Ovalbumin	43,000	4.633	0.408 (±0.005) [6]
4. Chymotrypsinogen A	25,000	4.398	0.511 (±0.006) [6]
5. Ribonuclease A	13,700	4.137	0.554 (±0.006) [5]
6. Aprotinin	6,500	3.813	0.705 (±0.010) [3]

Fig.A2.1 Molecular weight calibration of the Superose 12 column using protein standards (including BSA-dimer) (PBS, pH7.4, 25°C, 0.48ml/min)



The calibration could also be performed using HSA and the dimer of HSA as data points (Table A2.2). Figure 2.1 shows an example calibration which yielded the following parameters.

	points 1-6	points 2-6
slope	-0.327 (± 0.052)	-0.341 (± 0.083)
intercept	1.934 (± 0.234)	1.992 (± 0.361)
R^2	98.7%	98.3%
r	0.9935	0.9915

(p=0.05)

Molecular weight calibration was sensitive to changes in the chromatographic variables and the condition of the column. Therefore molecular weight calibration was performed routinely during assays.

A2.1.2. Calibration with MTX

A series of solutions of MTX were produced from a 20mg/ml stock solution in PBS (pH 7.4). The following concentrations were produced: 0.001, 0.002, 0.0025, 0.004 and 0.005mg/ml. 200 ul samples were injected; detection being at 300nm. All samples were repeated and all injections duplicated. The output from the detector was quantified by the following methods:-

1. Measurement of peak height from the chart recorder
2. Integration and peak height using an LDC CI10 integrator
3. Integration using the BBC microcomputer

Table A2.3 lists data obtained for calibration of Superose with MTX. Figure A2.2 shows the plot of a representative set of data. A further repeat was conducted by measurement of peak height of the same samples after overnight storage at 4°C (Table A2.4). The regression analysis (Figure A2.3) yielded the following data:

slope (ml/mg), 300nm = 29.3×10^3

intercept = -0.6

r = 0.99992

The mean retention time of MTX during this experiment was 49.27 min (± 0.14min, 0.29%) which represented a Kav valued in excess of 1 (c.1.2 - actual value not calculated since the void volume had not been determined).

The calibration experiment was repeated at 280nm and 370nm using a concentration range of 0.02 - 0.1g/ml MTX and injecting 50 ul samples. The results obtained are shown in Table A2.4. The reproducibility of the Superose system was investigated by assaying the same MTX sample (0.01mg/ml) repeatedly with the following result (detection at 300nm)

Mean peak height = 121mm

sd = 1.11 (n = 23)

RSD = 0.91%

Table A2.3:
Calibration of the Superose 12 column with MTX (at 300 nm) (PBS, pH 7.4, 25°C, 0.5 ml/min)

Sample concentration [mg/ml]	Measured peak height [mm]	CI10 Integration		BBC microcomputer Area under curve
		Peak area	Peak height [mm]	
0.001	30 (±0)	171.3 (±1.0)	1095 (±9.4)	3.354 (±0.067)
0.002	60 (±0)	355.0 (±4.7)	2229 (±12.5)	7.038 (±0.080)
0.0025	75 (±0.2)	447.0 (±1.0)	2794 (±18.6)	8.879 (±0.173)
0.004	119 (±1.0)	713.1 (±4.7)	4415 (±47.4)	14.316 (±0.067)
0.005	147 (±1.9)	898.3 (±4.6)	5564 (±64.0)	18.086 (±0.414)
Slope [ml/mg]	29.3×10^3 ($\pm 6.9 \times 10^3$)	181.1×10^3 ($\pm 2.6 \times 10^3$)	1111.4×10^3 ($\pm 22.0 \times 10^3$)	3.674×10^3 ($\pm 0.041 \times 10^3$)
Intercept	1.30 ±2.22	-8.2 ±8.3	-3.8 ±71.2	-0.319 ±0.134
Correlation [r]	0.99991	0.99997	0.99994	0.99998

(sd)
(95% confidence limit)
n = 2

Fig.A2.2 Calibration of the Superose 12 column with MTX (300nm, PBS pH7.4, 25°C, 0.5ml/min.)

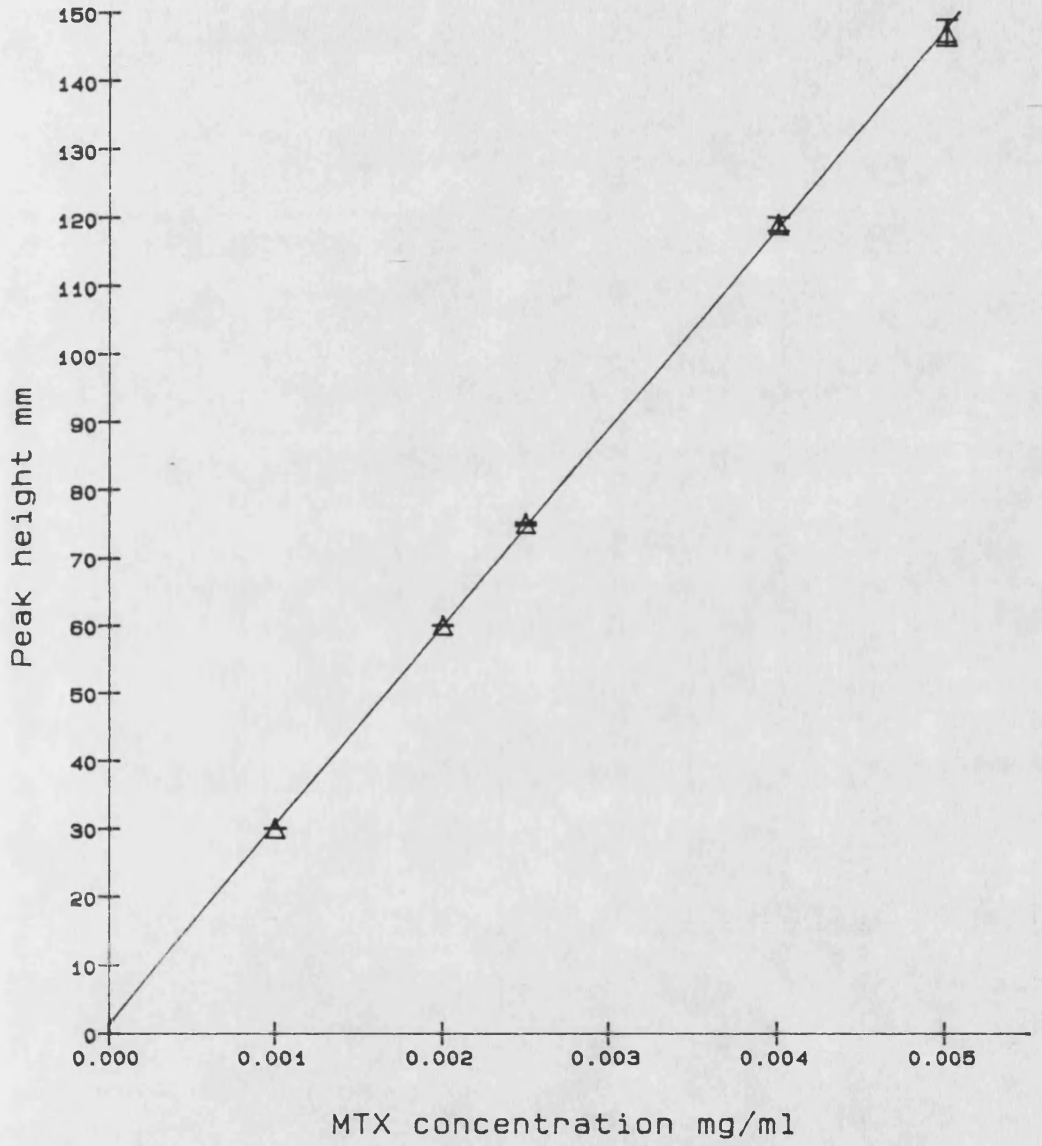
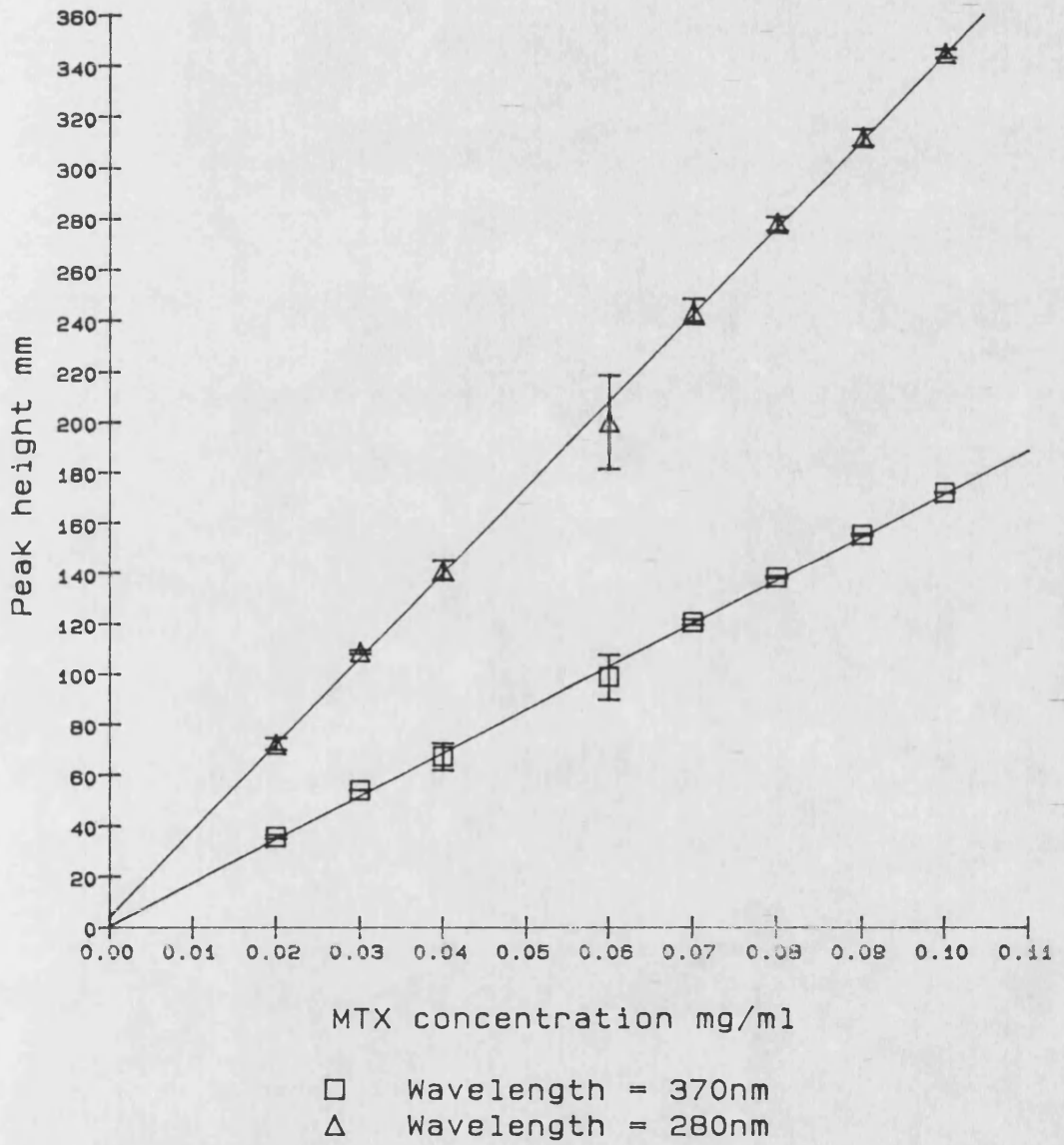


Table A2.4:
Calibration of the Superose 12 column with MTX (at 370 and 280 nm) (PBS, pH 7.4, 25°C, 0.5 ml /min)

Sample concentration mg/ml	WAVELENGTH			
	370 nm		280 nm	
	Run 1	Run 2	Run 1	Run 2
	Peak height/mm			
0.02	35.0	36.0	70.5	74.0
0.03	54.0	54.0	109.0	108.0
0.04	63.5	71.0	138.5	144.0
0.06	92.5	105.0	187.0	213.0
0.07	120.0	121.5	240.0	247.0
0.08	138.0	138.5	276.0	280.0
0.09	155.0	155.5	309.5	314.0
0.10	172.0	172.0	343.5	346.0
Slope/mm	1726.1 (±147.7)	1693.2 (±14.5)	3395.8 (±256.5)	3412.7 (±43.3)
Intercept	-1.98 (±9.89)	2.92 (±0.97)	1.26 (±17.18)	6.72 (±2.90)
r	0.996	1.000	0.997	1.000

(95% confidence limit)

Fig.A2.3 Calibration of the Superose 12 column with MTX (370nm, PBS pH7.4, 25°C, 0.5ml/min.)



A2.1.3. Calibration with HSA

a) A series of sample solutions were produced by dilution of a 10mg/ml stock solution. The following concentrations were produced in PBS pH 7.4: 0.1, 0.2, 0.3, 0.4, 0.5 and 1.0mg/ml. These gave a linear Beer-Lambert plot for peak height at 279nm with the following linear regression analysis:

slope (sd) = 5.37×10^{-1} (2.36×10^{-3}) ml/mg

intercept (sd) = 1.69×10^{-3} (1.20×10^{-3})

r = 0.99996

Peak area calibration was carried out by injecting 200ul samples onto the column the eluant being monitored at 280nm. Peaks were integrated using an LDC CI10 integrator. The results, means of repeat injections, are shown in Table A2.5. The plot of peak area against concentration yielded a straight line with the following regression analysis:—

slope (sd) = 1.890×10^{6} (5×10^{4})

intercept (sd) = -80.1×10^{3} (24.9×10^{3})

r = 0.99963

mean peak retention time (n=12) = 24.04 (0.03) min

b) The column was calibrated on a another occasion using 50ul samples and a Pye Unicam detector (280nm). The following peak heights were measured and corrected to 0.16 AUFS; all results were means of four injections (Table A2.6 and Figure A2.4).

Table A2.5:
Calibration of the Superose 12 column with HSA (at 279 nm)
(PBS, pH 7.4, 25°C, 0.5 ml/min)

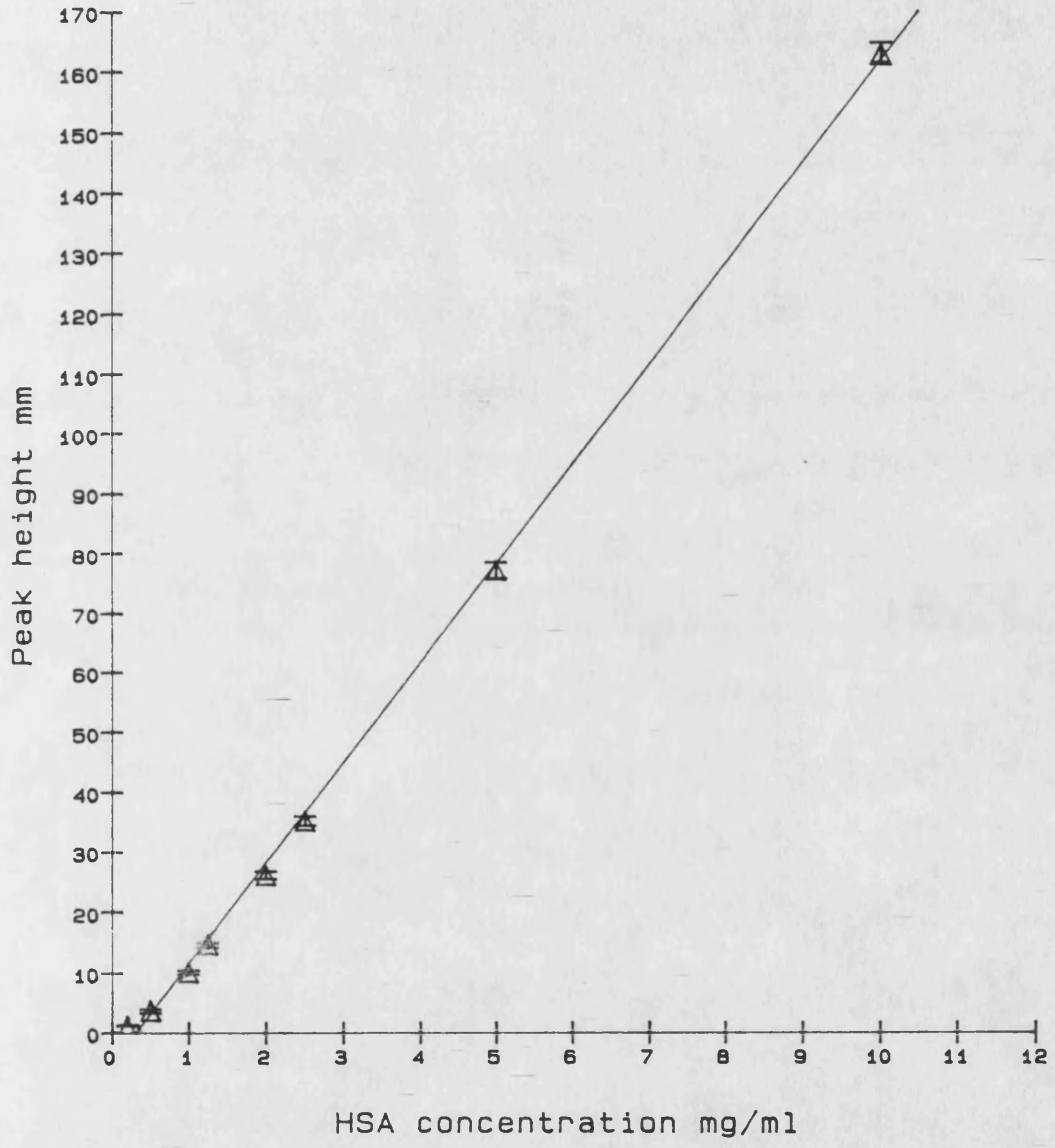
Sample concentration mg/ml	Peak Area x 10^3 (±sd) n = 2
0.1	123.7 (3.8)
0.2	291.9 (2.9)
0.3	485.7 (19.6)
0.4	677.2 (8.8)
0.5	847.5 (0.6)
1.0	1817.6 (15.3)

Table A2.6:
Calibration of the Superose 12 column with HSA (at 280 nm)
(PBS, pH 7.4, 25°C, 0.5 ml/min)

Sample concentration mg/ml	Peak height/mm (±sd) n = 4
0.2	1.0
0.5	3.5 (±0.3)
1.0	10.0 (±0.3)
1.25	14.5 (±0.4)
2.0	26.0 (±0.6)
2.5	35.0 (±0.73)
5.0	77.0 (±1.4)
10.0	163.0 (±1.8)

Slope = 16.78 [±0.53]
Intercept = -5.78 [±2.18]
r = 0.9995
[95% confidence limit]

Fig.A2.4 Calibration of the Superose 12 column with HSA (280nm, PBS pH7.4, 25°C, 0.5ml/min.)



The results of these calibrations indicated that the Superose 12 system was suitable for assaying human serum albumin since the retention time was found to be repeatable (and separate from the void volume) and linear calibration plots were obtained across the ranges 0.1 to 1mg/ml and 0.2 to 10mg/ml.

The chromatogram revealed a peak eluting prior to the main HSA peak (21.55min ± 0.28) which was due to the dimer of HSA and was present at a level of 11.3% (± 0.6) of the monomer peak (by peak height). The dimer peak was also found to exhibit linearity when detected at 280nm (r = 0.9995).

A2.1.4 Effect of dilution on assay of MTX-HSA

A conjugate of MCR 21 was produced and degraded at 37°C for twelve hours after which time the level of free MTX present was 3.7%. The solution was removed from the high temperature environment and diluted with PBS pH 7.4 and assayed using the Superose column at 300nm to determine MTX-HSA by peak area and peak height. Data is presented in Table A2.7.

A2.2 Calibration of the TSK G3000 PWxl column

A2.2.1 Molecular weight calibration

The calibration was performed using a variety of protein standards under the following conditions:

Table A2.7:
Calibration of the Superose 12 column with MTX-HSA conjugate (including free MTX) (PBS, pH 7.4, 25°C, 0.5 ml/min)

	Conjugate		MTX	
Sample Concentration expressed as HSA mg/ml	Dimer peak area x 10^5	Monomer peak area x 10^5	Peak area x 10^4	Peak height/mm
0.171 (33.3)	13.2 (27.7)	41.8 (31.2)	13.0 (31.9)	25.0 (34.3)
0.257 (50.0)	21.3 (44.7)	63.6 (47.5)	20.3 (49.8)	37.0 (50.7)
0.342 (66.67)	29.4 (61.6)	85.9 (64.1)	27.4 (67.2)	48.5 (66.4)
0.428 (83.3)	38.5 (80.7)	110.2 (82.2)	33.5 (82.1)	59.5 (81.5)
0.513 (100.0)	47.7 (100.0)	134.0 (100.0)	40.8 (100.0)	73.0 (100.0)
Retention time/min	22.58 [±0.04]	25.46 [±0.06]	50.93 [±0.08]	
Regression A	-4.48	-5.35	-0.54	1.17
B	100.8	270.2	80.5	138.6
r	0.9995	0.9997	0.9996	0.9994

(expressed as % of maximum value)

Flow rate:	0.78ml/min
Mobile phase:	Phosphate buffered saline pH7.4
Column Temperature:	25°C ± 0.5°C
Detection:	280nm, Gilson 113
Sample preparation:	dissolved in mobile phase and filtered 0.2um
Sample volume:	20ul
Column volume:	3.31ml (pre column) + 14.33ml (main)
	Vt = 17.64ml, Vo = 7.02ml

Representative data for calibration are shown in Table A2.8 and Figure A2.5. The results from calibration 2 were the mean values (±sd) from six different chromatograms run over a three day period.

The regression analysis was as follows:

Slope = - 0.419 (0.081) (95% confidence limits)

Intercept = 2.140 (0.342)

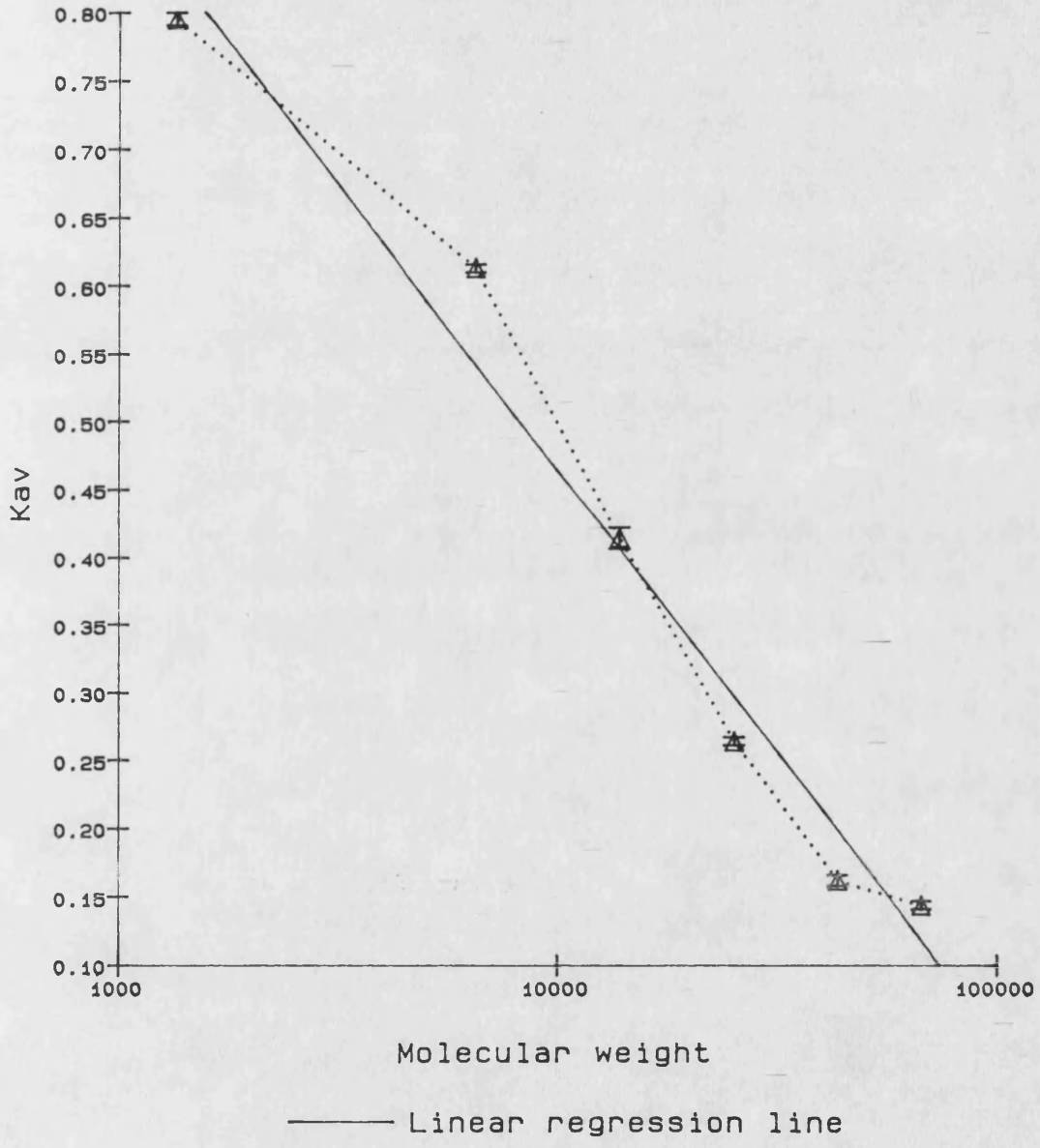
R^2 = 97.3%, r = - 0.986

When the BSA dimer (134,000), Kav = 0.099, was included in the calibration the plot exhibited the classical sigmoid curve suggesting that the TSK G3000PWxl column had a useful separation range between about 5,000 and 50,000 Daltons. The regression line (excluding the dimer) (Figure A2.5) gave a reasonable fit (r = 0.986). It was thought that the TSK G3000 column could be used for molecular weight estimation within the range 1,500 - 70,000 Daltons (correlation for 6,500 - 43,000 = 0.997). However it was noted that the performance of the Superose column was superior over this range.

Table A2.8:
Molecular weight calibration of the TSK column using protein standards (PBS), pH 7.4, 25°C, 0.78 ml/min)

Sample	Molecular Weight (MW)	log MW	Elution volume Ve/ml	Run 1 Kav n = 1	Run 2 Kav n = 6 (±sd)
BSA	67,000	4.826	8.52	0.143	0.144 (±0.003)
Ovalbumin	43,000	4.634	8.79	0.168	0.162 (±0.004)
Chymotrypsinogen A	25,000	4.398	9.82	0.267	0.264 (±0.003)
Trypsin	23,800	4.377	10.08	0.291	-
Ribonuclease	13,700	4.137	11.31	0.408	0.414 (±0.008)
Aprotinin	6,500	3.813	13.52	0.619	0.613 (±0.003)
Vitamin B12	1,353	3.131	15.37	0.795	0.795 (±0.005)

Fig.A2.5 Molecular weight calibration of the TSK G3000PWx1 column using protein standards (280nm, PBS pH7.4, 25°C, 0.78ml/min.)



A2.2.2. Calibration with MTX

A series of concentrations of MTX were produced in PBS pH7.4 from a stock solution (20mg/ml) and 20ul samples assayed using the TSK column.

Mobile phase = PBS pH7.4

Flow = 0.749ml/min (0.75ml/min in run 2).

The output was monitored at 303nm and also logged by computer to determine the peak areas. Sample '2' represented a second run with samples produced from a fresh stock solution; each data point was the mean of 2 samples (Table A2.9). The mean retention times of MTX were:

Sample 1: 24.76 min ($\pm$0.06) (n = 5)

Sample 2: 24.86 min ($\pm$0.03) (n = 8)

This equated to a Kav of approximately 1.09 indicating that MTX was also adsorbed to the TSK column. The results (the mean values of which are represented graphically in Figure A2.6) showed that the TSK system provided both a repeatable and a reproducible assay for MTX across the range studied and that the peak height and peak area methods of analysis were equally valid.

A2.2.3 Calibration with HSA

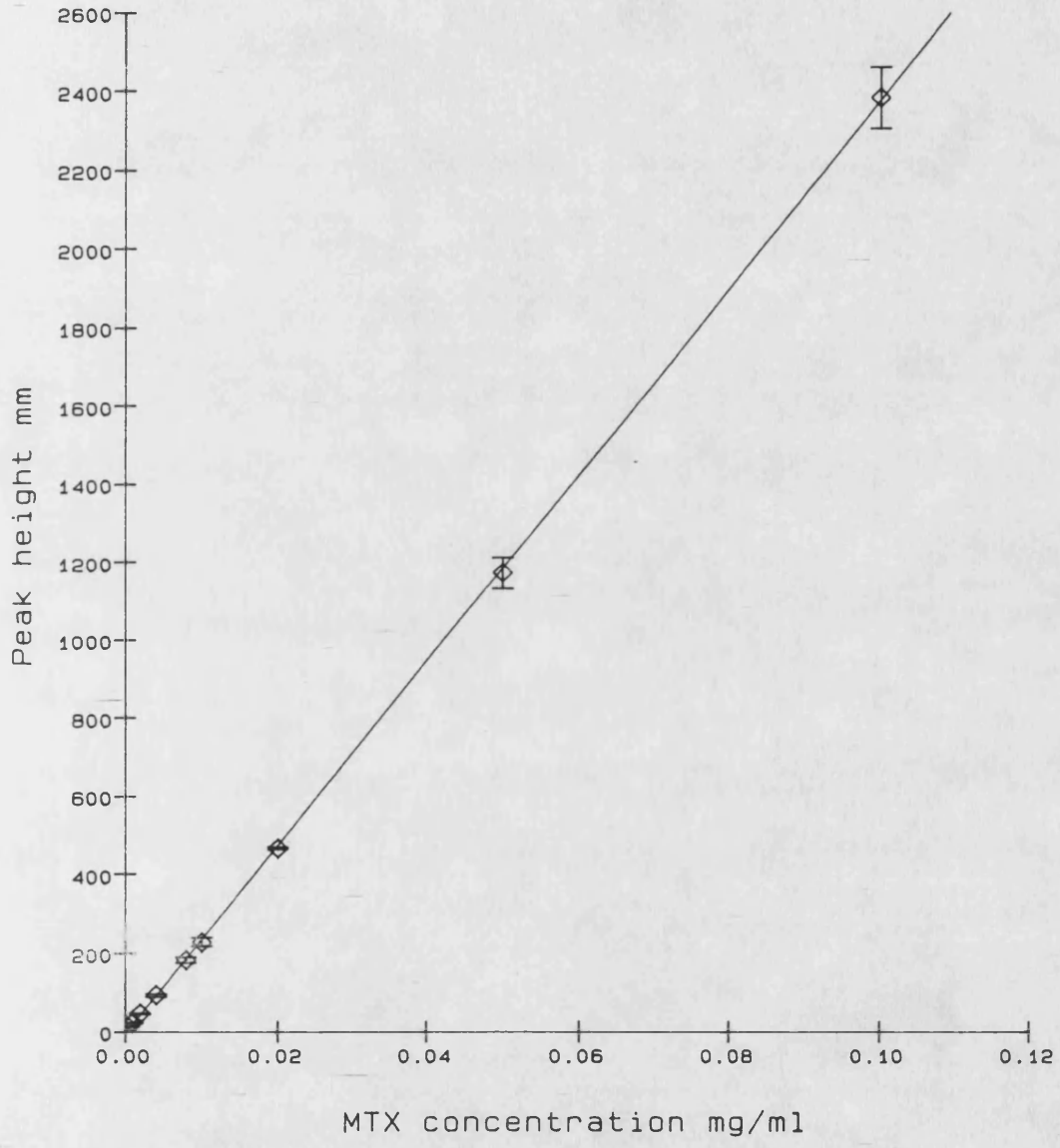
A stock solution was prepared by dissolving HSA in PBS pH 7.4 to give a final concentration of 5.0mg/ml. Aliquots from this solution were diluted

Table A2.9:
Calibration of the TSK column with MTX (at 303 nm) (PBS pH 7.4, 25°C, 0.75 ml/min)

Sample concentration mg/ml	Sample 1		Sample 2	
	Mean peak height (mm) corrected to 0.02 aufs	Mean peak area corrected to 0.02 aufs	Mean peak height (mm) corrected to 0.02 aufs	Mean peak area corrected to 0.02 aufs
0.0005	12.8	1.2744	11.3	1.2034
0.001	23.4	2.3865	21.4	2.1904
0.002	46.8	4.8702	45.0	4.7853
0.004	95.3	9.8470	90.0	9.5445
0.008	188.0	19.2514	177.5	18.6755
0.01	235.0	23.3176	221.0	22.9562
0.02	467.5	-	465.0	47.8065
0.05	1200.0	118.7430	1143.8	116.6913
0.1	2440.0	244.1540	2330.0	233.4560
Regression analysis				
Slope	24373 (±220)	2432.7 (±30.8)	23277 (±191)	2334.3 (±11.2)
Intercept	-6.2 (±7.9)	-0.4 (±1.2)	-5.1 (±7.3)	0.1 (±0.4)
R^2	100.0%	100.0%	100.0%	100.0%
r	1.000	1.000	1.000	1.000

(95% confidence limit)

Fig.A2.6 Calibration of the TSK G3000PWx1 column with MTX (303nm, PBS pH7.4, 25°C, 0.75ml/min.)



to give a range of concentrations, 20ul samples of which were assayed at 279nm after being chromatographed on the TSK column using the following conditions:

Mobile phase: PBS pH 7.4

Flow rate: 0.745ml/min

The results, shown in Table A2.10, gave the regression analysis below:

Slope = 172.9 ($\pm$4.4)

Intercept = 1.80 ($\pm$10.93)

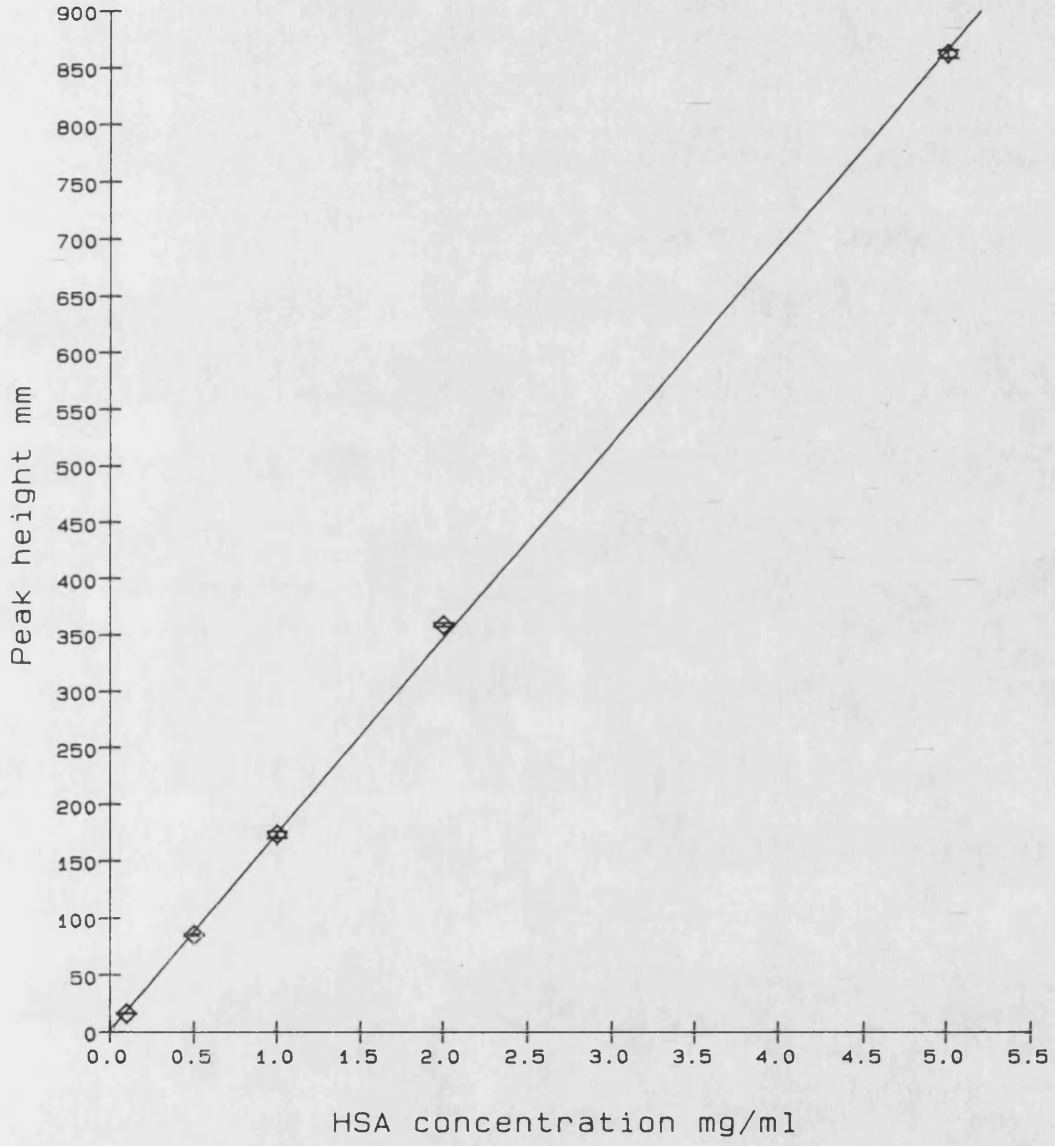
R^2 = 100.0%, r = 1.000

Representative results are shown graphically in Figure A2.7.

Table A2.10:
Calibration of the TSK column with HSA (at 279 nm)
(PBS, pH 7.4, 25°C, 0.75 ml/min)

HSA Concentration mg/ml	Mean peak height (mm) corrected to 0.04 auf (±sd) n = 2
0.1	16.4 (±0.0)
0.5	85.1 (±0.5)
1.0	173.0 (±2.8)
2.0	359.0 (±1.4)
5.0	862.5 (±3.5)

Fig.A2.7 Calibration of the TSK G3000PWx1 column with HSA (280nm, PBS pH7.4, 25°C, 0.75ml/min.)



APPENDIX A3: ISOLATION AND CHARACTERISATION OF LYSOSOMAL ENZYMES FROM THE RAT LIVER

Appendix A3 The extraction and characterisation of lysosomal enzymes from rat liver

Lysosomal enzymes were extracted from rat liver using the principles described by Trouet (1974). Male Wistar rats (280-320g) were injected intraperitoneally with 3ml 20%w/v Tyloxapol solution in Milli Q water. The rats were killed after 4 days, their livers removed and homogenised at 4°C in 0.25M sucrose. The homogenate was centrifuged at 2000 rpm for 15 minutes, the supernatant removed and recentrifuged at 12,000 rpm for 30 minutes. The pellet was suspended in 45%w/v sucrose solution and then centrifuged at 25,000 rpm for 2 hours on a sucrose gradient composed of equal parts of 45%, 35.5% and 14.5% sucrose solutions. A distinct band of lysosomal material was present at the interface of the 14.5% and 35.5% sucrose layers. This fraction was removed using a Pasteur pipette, its volume noted, and stored at -18°C.

The enzyme activity was monitored using Bz-Phe-Val-Arg-NAp as substrate and the reaction monitored by change in UV absorbance at 410nm in citrate -phosphate buffers at 35°C. The following formula was used in activity experiments.

tritosome suspension	0.10 ml
1%w/v Synperonic OP10	0.02 ml
EDTA 50mM	0.02 ml
GSH 250mM	0.02 ml
double strength buffer	0.50 ml
water	0.32 ml
Bz-Phe-Val-Arg-NAp (4x10^{-3}M in DMF)	0.02 ml

	1.00 ml

Typical data generated using the above formula is shown in the table below.

Incubation time (min) at pH 5.0, 35°C	change in absorbance at 410nm
5	0.015
10	0.062
15	0.129
20	0.183
25	0.289
45	0.415
60	0.710
120	1.082
180	1.270

The effect of pH on the activity of the same batch of tritosomes was studied after 10 weeks storage of the enzyme preparation at -18°C. Data obtained is shown in the following table.

pH	mean change (sd) in absorbance at 410nm after 20 min at 35°C
4.2	0.149 (0.003)
4.6	0.149 (0.001)
5.0	0.181 (0.003)
5.6	0.208 (0.009)
6.0	0.215 (0.005)
6.6	0.184 (0.006)
7.0	0.287 (0.017)
8.1	0.053 (0.002)

This indicated that the enzyme preparation had substantial activity between pH 4 and 7 but lower activity at pH 8. Storage of the enzyme preparation for 10 weeks had no adverse effect on its activity. A blank system containing no tritosomes but replacing their volume by 20% sucrose demonstrated no change in absorbance at 410nm over the 20 min incubation period.

The effect of reduced glutathione on the degradation of Bz-Phe-Val-Arg-NAp was investigated using a lower substrate concentration (as before but using a stock solution of 1.5×10^{-3}M substrate in DMF). Data obtained is shown below.

concentration of reduced GSH	change in absorbance at 410nm after 20 min at 35°C, pH 5.0
0	0.008
5 mM	0.063
10 mM	0.087
50 mM	0.078

The above data showed that degradation of the substrate at pH 5 was thiol dependent and that there was no substantial change in degradation rate within a concentration range of 5-50 mM GSH. A concentration of 5mM GSH was adopted for degradation experiments described in Chapters 3 and 4.

The effect of surfactant concentration on enzyme activity was also investigated (see below).

volume 1% Synperonic OP10 added to incubation mixture	change in absorbance at 410nm after 20 min at 35°C, pH 5.0
0.02 ml	0.084
0.20 ml	0.060

There was no advantage to be gained by increasing the surfactant concentration above 0.02 ml therefore the lower surfactant concentration was adopted as standard. The surfactant was present to allow solubilisation of the lysosomal membranes and release of enzymes. It was not clear what was the effect of freeze-thawing on the enzyme preparation

but the turbidity of the suspension was thought to be lower after freeze-thawing. The enzymes may have been present in solution after freeze-thawing making the addition of extra surfactant unnecessary.

Assay of surfactants used in the isolation of tritosomal enzymes and screening of those employed in the lysosomal experiments

The surfactant which has been used in the isolation of lysosomal enzymes was a cross-linked octylphenol ethoxylate, Triton WR1339 (Trouet, 1974). A surfactant with a similar composition ie Tyloxapol (obtained from Sigma) was employed in this assay. Triton X-100 has been used for tritosomal degradation studies since it is necessary to solubilise the lysosomal vesicle to release the enzymes. In this study surfactants with similar structures and HLBs to Triton X-100 were selected and assayed in order to discover which one would interfere least with the FPLC assay.

The surfactant solutions were produced by dissolving sufficient surfactant in pH5.6 buffer to produce at 10% w/v solution (all of which were found to be clear). A 1 in 50 dilution was then produced (ie 0.2%w/v) and 20ul samples applied to the Superose 12 column (mobile phase pH7.4, detection 303nm). Peaks resulting from the surfactants are described in the Table below.

	Retention time (mins)		
Peak No	1	2	3
Tyloxapol	-	24.5 (large)	33 + 34.5
Lutensol AO8	14	24	33 + 34.5
Synperonic NP13	14	24.5	33 + 34.5
Synperonic OP10	-	-	33 + 35

Synperonic OP10 was selected as the solubilizing surfactant since it only exhibited peaks at around 34 minutes which were probably due to the buffer system used. Other surfactants gave a peak eluting with the void volume of the column which may have been due to micellar material.

APPENDIX A4: EXPERIMENT TO CONFIRM THAT MICROBIOLOGICAL SPOILAGE WAS NEGLIGABLE DURING IN VITRO DEGRADATION STUDIES

Appendix A4 Experiment to confirm that microbiological spoilage of MTX-HSA conjugates was negligible during *in vitro* degradation studies

The experimental procedure employed in the formation of the conjugate involved the use of clean/sterile glassware (dry heat, 160°C for a minimum of two hours) wherever possible. Solutions were filtered through a sterile 0.2um (or 0.45um) membrane filter into the sterile glass containers/vials. However it was clear that microbial contamination of HSA or MTX-HSA solutions resulted in rapid degradation of the HSA. Microbial growth was observed in non-sterile HSA solutions stored under ambient conditions for 24 hours. Assay of such a system showed that considerable quantities of free MTX were released from MTX-HSA in the presence of bacteria. Thus to confirm the sterility of the samples use for *in vitro* degradation studies, an experiment was set up which included sterility checks on the actual samples which were analysed by FPLC.

The conjugation reaction was performed under the normal conditions used for the active ester method and separated on a Sephadex column (5.13ml injected, 100ml collected) in pH7.4 buffer then sterile filtered into sterile 2ml vials. The MCR was found to be 19.4 (MTX concn. = 68.4 x 10^{-3}mg/ml).

Sterility checks were performed, at various times over a 48 hour incubation period (at 37°C), in the following manner using aseptic technique:-

1. The rubber cap of the vial was swabbed with 70% alcohol and then a 1ml sample withdrawn using a sterile syringe/hypodermic needle.

2. The sample was emptied on to a sterile 0.2um filter contained in a Millipore filtration unit (vacuum on), the filter had previously been rinsed with a few millilitres of sterile water. The sample/filter were then rinsed with about 100ml of sterile water.

3. The filter was then transferred aseptically to an overdried nutrient agar plate.

4. The plate was incubated at 37°C for 48 hours before any colonies were counted. The plate was then stored at 25°C for 72 hours to check for the presence of moulds.

The samples were then analysed on the Superose 12 column following incubation at 37°C, sterility tested samples were assayed within 30 minutes of testing. The samples were analysed for molecular weight distribution using the integration programme and for release of free MTX by peak height. Data obtained is shown below.

Incubation time at 37°C	sterility test	peak 1 (dimer)		peak 2 (HSA)		% free MTX
		area(%)	MW(x10^{-3})	area(%)	MW(x10^{-3})	
0	NG	15.1	134	73.6	68.9	-
1hr 10m	-	16.5	141	73.5	69.7	0.06
2hr 49m	NG	16.9	140	73.1	69.3	0.13
4hr 29m	-	16.4	139	73.5	68.7	0.20
6hr 14m	-	16.5	140	73.7	69.2	0.24
7hr 24m	-	16.8	135	74.2	67.6	0.29
19hr 44m	NG	16.8	140	72.9	68.8	0.55
24hr 44m	NG	-	-	-	-	0.64
31hr 26m	-	15.9	-	73.2	-	0.73
47hr 44m	NG	-	-	-	-	0.88
51hr 54m	-	-	-	-	-	0.91
66hr 09m	-	-	-	-	-	0.98

(NG, no growth; -, not tested)

The reaction mixture was also sterility tested after 3 days at 25°C with the result that no growth occurred.

A further sterility experiment was conducted using an ECDI produced conjugate.

All glassware was heated at 160°C for at least 2 hours prior to use to ensure sterility. The MTX, ECDI and HSA were weighed into the appropriate volumetric flasks, as were the buffer salts to which sterile water was added aseptically. The buffer solutions were then filtered (0.2um

sterile) into the MTX, ECDI and HSA volumetric flasks in a laminar flow cabinet. The required volumes of each solution were mixed and then filtered (0.2um) into two vessels (HSA:MTX:ECDI = 1:116.2:172.5). One of the mixtures was then inoculated with a colony of Pseudomonas (a potential contaminant). The reaction mixtures were then incubated at 25°C overnight.

The inoculated sample (RM21 INN) was filtered on to a Sephadex G50 column (1.04ml sample) after incubation for 13.5 hours and the resulting 25ml sample sample filtered and analysed as normal.

The sterile sample (RM21) was desalted after storage for 36 hours (5.13ml fractionated and collected in 100ml) then sterile filtered and analysed on the Superose 12 column. The MCR was found to be 20.0 (reaction mixture HSA:ECDI:MTX = 1:116.2:172.5)

RM21INN		RM21	
time post-fractionation	% free MTX	time post-fractionation	%free MTX
1hr 00m	0.15	1hr 30m*	0.21
9hr 45m	1.99	3hr 40m*	0.27
17hr 10m*	3.33	9hr 10m	0.90
21hr 55m*	3.93	13hr 50m*	1.30
45hr 55m	5.91	31hr 10m	3.24
47hr 00m	5.99	32hr 14m	3.45
		34hr 14m	3.61

(* - sterility tested all showing no growth)

The conjugation mix from RM21INN was also tested and found to be heavily contaminated. All filtered samples were found to be sterile.

The release of MTX from the conjugates was as expected and could not be explained by microbial spoilage of the MTX-HSA since all the samples tested were sterile. The addition of microorganisms to the reaction mixture did not affect the integrity of the conjugate although the release of MTX was higher from the inoculated sample. This was thought to be due to the shorter reaction time used in the latter case.

APPENDIX A5: THE EFFECT OF SODIUM AZIDE ON THE ENZYMATIC DEGRADATION OF MTX-HSA AND MTX-COPOLY(AMINO ACID) CONJUGATES

Appendix A5 The effect of sodium azide on the enzymatic degradation of copolymer and HSA-MTX conjugates

The purpose of this experiment was to determine whether the addition of sodium azide (0.02% w/v) to the mobile phase resulted in any alteration of the degradation profile of the conjugates. Sodium azide had been incorporated into the phosphate buffer in order to minimize the risk of microbial spoilage of either the samples or the column employed for separation and analysis.

i) L-copolymer

The sample of L-copolymer-MTX conjugate used in this experiment was that described in section 4.5; this was in solution (0.125mg/ml) in PBS (pH 7.4) containing azide. 7.5ml of this solution was freeze dried and then reconstituted with 1.2ml water and 1ml separated on a Sephadex G15 column, using PBS-(pH7.4 no azide) as the mobile phase, the sample being collected in a volume of 10ml (0.078mg/ml). The solution was then sterile filtered and 250ul samples placed in vials to which were added 25ul aliquots of two of the following:- trypsin solution 1mg/ml, PBS pH7.4 or sodium azide in PBS (2.4mg/ml) in order to produce the following solutions.

RM35A - copolymer/MTX, no trypsin, no azide

RM35B - copolymer/MTX, no trypsin + azide 0.02%

RM35C - copolymer/MTX, trypsin, no azide (Cop:try = 0.78:1)

RM35D - copolymer/MTX, trypsin + azide 0.02% (Cop:try = 0.78:1)

The samples were then sealed, mixed and incubated at 37°C for 10 and 24 hours prior to assay on the TSK system.

The results for the samples without trypsin ie RM35 A and B showed that there was no alteration in the molecular weight of the main peak (36,000) but the sample containing azide exhibited a few low molecular weight peaks causing the proportion of the main peak to drop from 99% without azide to 92% in its presence.

The incorporation of trypsin brought about the characteristic dismantling of the conjugate molecule into various molecular weight fractions. A slight reduction in activity was evident in the presence of sodium azide.

Sample	Molecular weight ($x10^{-3}$)				
	27	15	8	3.5	<1.5
RM35C-10H	15.6	13.3	18.9	24.3	27.8
RM35D-10H	17.4	15.3	20.1	22.0	25.2
RM35C-24H	13.5	15.1	18.1	24.5	28.8
RM35D-24H	15.5	15.1	19.0	24.3	26.2

ii) HSA

The conjugate reaction mixture, which had been previously frozen, was separated on a Sephadex column (1.04ml fractionated and collected in 25ml) using PBS pH7.4 (no azide) as mobile phase. The sample (MCR = 9.9) was then sterile filtered and 0.5ml aliquots mixed with 50ml trypsin solution (4mg/ml) or 50ul PBS or 50ul PBS + azide (2.4mg/ml) to produce the following samples:-

RM36A - HSA/MTX no trypsin, no azide

RM36B - HSA/MTX no trypsin + azide 0.02% w/v

RM36C - HSA/MTX trypsin, no azide molar HSA: Try = 1:2.7

RM36D - HSA/MTX trypsin + azide 0.02%w/v molar HSA: Try = 1:2.7

The vials were sealed, mixed and incubated at 37°C for 10 and 24 hours and frozen before assay on the TSK system

Sample in the absence of trypsin

Sample	Molecular weight $x10^{-3}$			%free MTX
	70	50	MTX	by peak height
RM36A-10H	7.0	87.5	1.5	2.0
RM36B-10H	6.7	86.8	1.5	2.0
RM36A-24H	6.2	87.2	2.2	3.1
RM36B-24H	6.0	88.4	1.9	3.0

Sample in the presence of trypsin

Sample	Molecular weight $x10^{-3}$					
	48	24	12	7.5	4.5	<1.5
RM36C-10H	25.5	28.7	11.3	6.7	10.2	15.8
RM36D-10H	28.5	27.5	10.4	6.5	10.4	15.2
RM36C-24H	23.2	27.1	10.9	7.1	9.9	19.9
RM36D-24H	21.1	28.3	11.8	6.3	10.2	20.5

A comparison of the overall molecular weight distribution was determined with a fixed baseline:-

Sample	MW $x10^{-3}$	Mw/Mn
RM36A-10H	51.6	2.4
RM36B-10H	51.9	2.4
RM36C-10H	22.8	6.1
RM36D-10H	24.5	6.5

The results showed that the addition of sodium azide had very little effect on either release of free MTX in buffer solution or the degradation of the samples in the presence of trypsin.

APPENDIX A6: PHYSICAL AND SPECTROSCOPIC PROPERTIES OF METHOTREXATE

Appendix A6 Physical and spectroscopic properties of MTX

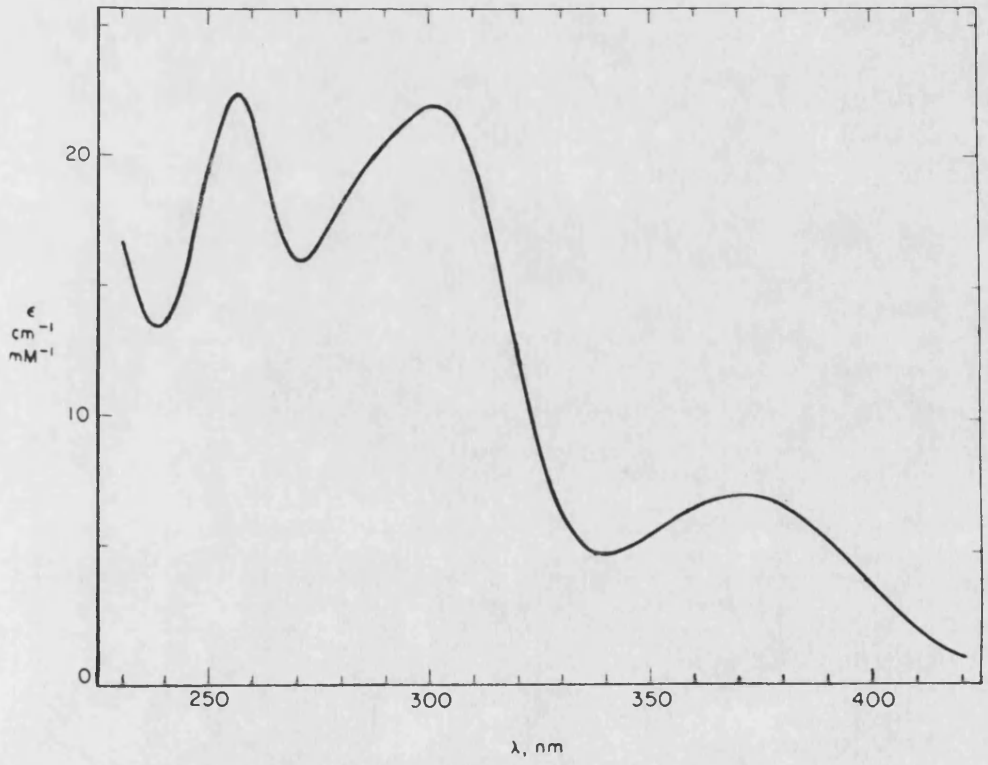
The chemical structure of MTX is shown in section 1.4.2

acidic dissociation constants (25°C, I = 0.1M) (Poe, 1977)

	pKa
α-carboxyl	3.36
γ-carboxyl	4.70
N(1)	5.71
N(5)	<-1.5
N(10)	0.50

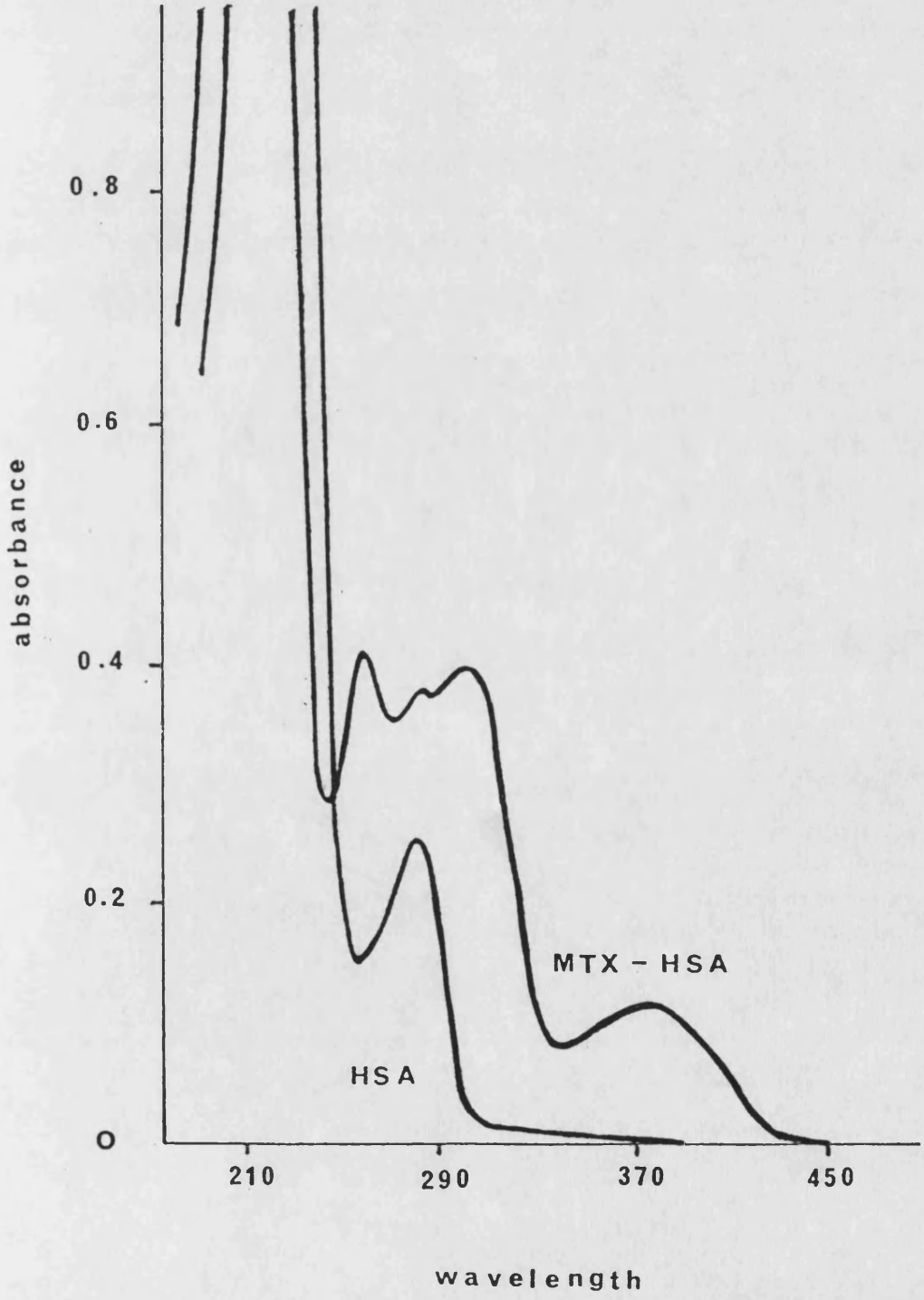
Molecular weight = 454.4

UV λ max (aqueous alkali): 258nm, 302nm and 370nm. A typical spectrum is shown below.



Absorption coefficients were determined in phosphate buffered saline pH 7.4 using a range of concentrations (0.001-0.1mg/ml) (Beer-Lambert linear r = 1.000, E1%/1cm = 162.4, molar absorption coefficient = 7380,370nm)

The UV spectrum for MTX_{20}-HSA is displayed with that for HSA at an equivalent protein concentration of 5mg/ml.



APPENDIX A7: COMPUTER PROGRAMMES FOR COLLECTION AND ANALYSIS OF DATA

```
LI.
   10 REM ****************************************
   20 REM *  PROGRAM FOR SAVING CHROMATOGRAPHIC  *
   30 REM *          DATA ON DISC                *
   40 REM *                                      *
   50 REM *  R J MARRIOTT AND C W POUTON         *
   60 REM ****************************************
   70 CLS
   80 PRINT"WARNING"
   90 PRINT
  100 PRINT"HAVE YOU RENAMED PREVIOUS FILES?"
  110 PRINT
  120 PRINT"IF NOT BY CONTINUING WITH THIS"
  130 PRINT"PROGRAM YOU WILL DELETE THEM"
  140 PRINT
  150 PRINT
  160 DIM DS(1000)
  170 VDU 15
  180 DT=100
  190 INPUT "ENTER DATA COLLECTION TIME (min) "DT
  200 ST%=DT*6000/1000
  210 INPUT"ENTER THE NUMBER OF INJECTIONS (1-20)"NC
  220 FOR K%=1 TO NC
  230 PROCSTART
  240 PROCNAME
  250 PROCDATACOL
  260 NEXT
  270 PRINT "FINISHED SERIES OF "NC" INJECTIONS"
  280 STOP
  290 END
  300 :
  310 DEF PROCDATACOL
  320 FOR I%=1 TO 1000
  330 CT%=TIME
  340 AV=0
  350 FOR J%=1 TO 100
  360 AV=AV+ADVAL(2)
  370 NEXT
  380 AV%=AV/100
  390 DS(I%)=AV%/34900
  400 PRINT I%;"        ";DS(I%);"  ";K%
  410 PRINT£X,I%,DS(I%)
  420 IF TIME<(CT%+ST%) THEN 420
  430 NEXT
  440 CLOSE£X
  450 ENDPROC
  460 :
  470 DEF PROCNAME
  480 ON K% GOTO 490,510,530,550,570,590,610,630,650,670,690,
  770,790,810,830,850,870
  490 X=OPENOUT "INJ1"
  500 GOTO 250
  510 X=OPENOUT "INJ2"
  520  GOTO 250
  530 X=OPENOUT "INJ3"
  540  GOTO 250
  550 X=OPENOUT "INJ4"
  560 GOTO 250
```

```
570 X=OPENOUT "INJ5"
580 GOTO 250
590 X=OPENOUT "INJ6"
600 GOTO 250
610 X=OPENOUT "INJ7"
620 GOTO 250
630 X=OPENOUT "INJ8"
640 GOTO 250
650 X=OPENOUT "INJ9"
660 GOTO 250
670 X=OPENOUT "INJ10"
680 GOTO 250
690 X=OPENOUT "INJ11"
700 GOTO 250
710 X=OPENOUT "INJ12"
720 GOTO 250
730 X=OPENOUT "INJ13"
740 GOTO 250
750 X=OPENOUT "INJ14"
760 GOTO 250
770 X=OPENOUT "INJ15"
780 GOTO 250
790 X=OPENOUT "INJ16"
800 GOTO 250
810 X=OPENOUT "INJ17"
820 GOTO 250
830 X=OPENOUT "INJ18"
840 GOTO 250
850 X=OPENOUT "INJ19"
860 GOTO 250
870 X=OPENOUT "INJ20"
880 GOTO 250
890 ENDPROC
900 :
910 DEF PROCSTART
920 PRINT"TO START CHROMATOGRAPHIC RUN PRESS  G"
930 START$=GET
940 IF START$="G" THEN 950 ELSE 920
950 ENDPROC
```

```
LI.LI.
   10 REM ****************************************
   20 REM *  PROGRAM TO RETRIEVE AND ANALYSE      *
   30 REM *  SIZE EXCLUSION CHROMATOGRAPHIC DATA  *
   40 REM *                                       *
   50 REM *  R J MARRIOTT AND C W POUTON          *
   60 REM ****************************************
   70 *FX114,0
   80 MODE0
   90 @%=10
  100 DIM DS(1000),STY(20),STX(20),SP%(10),EP%(10),PH%(10),CUT%(6),P(6)
  110 DIM peak$(5):peak$(1)="FIRST":peak$(2)="SECOND":peak$(3)="THIRD"
  120 peak$(4)="FOURTH":peak$(5)="FIFTH"
  130 DIM cut$(6):cut$(1)="FIRST":cut$(2)="SECOND":cut$(3)="THIRD"
  140 cut$(4)="FOURTH":cut$(5)="FIFTH":cut$(6)="SIXTH"
  150 VDU15
  160 SP=0.5
  170 SP%(1)=250:SP%(2)=250:SP%(3)=250:SP%(4)=250:SP%(5)=250:SP%(6)=250
  180 EP%(1)=750:EP%(2)=750:EP%(3)=750:EP%(4)=750:EP%(5)=750:EP%(6)=750
  190 PH%(1)=100:PH%(2)=100:PH%(3)=100:PH%(4)=100:PH%(5)=100
  200 CUT%(1)=100:CUT%(2)=100:CUT%(3)=100:CUT%(4)=100:CUT%(5)=100:CUT%(6)=100
  210 P1=1:P2=2:P3=3:P4=4:P5=5:P6=6
  220 P=50
  230 A=-3.658324
  240 B=6.102622
  250 TN=15.56
  260 VT=23.4
  270 ATEST=1
  280 DT=65
  290 ST%=DT*6000/1000
  300 VDU26
  310 CLS
  320 *FX15,1
  330 PROCRETRIEVE
  340 PROCINF
  350 PROCCF
  360 PROCDESC
  370 MODE0
  380 CLS
  390 CLG
  400 VDU 26
  410 PROCANAL
  420 *FX15,1
  430 MODE0
  440 PRINT "DO YOU WISH TO ANALYSE THE SAME DATA AGAIN?Y/N";:INPUT Y$
  450 IF Y$="Y" OR Y$="y" THEN 370 ELSE GOTO 460
  460 PRINT"HAVE YOU FINISHED ? Y/N"
  470IF GET=78 THEN 330
  480 END
  490:
  500 DEF PROCINF
  510 CLS
  520 FLAG%=0
  530 PRINT"OPTIONS AVAILABLE AT THIS STAGE"'
  540 PRINT"f2   -   CARRY OUT CALIBRATION"'
  550 PRINT"f3   -   CONTINUE WITH RUN"'
  560 PROCKEY
  570 IF FLAG%=0 THEN 560
```

```
 580 IF FLAG%<>1  THEN 510
 590 ENDPROC
 600:
 610 DEF PROCKEY
 620 *FX15,1
 630 IF INKEY(-115)=-1 THEN PROCCALIB
 640 IF INKEY(-116)=-1 THEN FLAG%=1
 650 ENDPROC
 660:
 670:
 680 DEF PROCCALIB
 690 INPUT"ENTER FLOW RATE (ml/min) "SP
 700 INPUT"ENTER DEXTRAN (MW=2,000,000) ELUTION TIME (min) "TN
 710 INPUT"ENTER TOTAL VOLUME OF COLUMN (ml) "VT
 720 INPUT"ENTER NO. OF CALIB. POINTS "N%
 730 FOR I%=1 TO N%
 740 PRINT"MOL. WT. "I%
 750 INPUT MLW
 760 STY(I%)=LOG(MLW)
 770 PRINT"ELUT. TIME (min) "I%
 780 INPUT STX(I%)
 790 NEXT
 800 FOR I%=1 TO N%
 810 STX(I%)=(STX(I%)*SP-TN*SP)/(VT-TN*SP)
 820 NEXT
 830:
 840 PROCLINREG
 850 PRINT"PRESS SPACE TO CONTINUE"
 860 IF GET<>32 THEN 860
 870 FLAG%=2
 880 ENDPROC
 890 DEF PROCDESC
 900 INPUT"ENTER A NAME AND/OR SHORT DESCRIPTION OF YOUR SAMPLE "D$
 910 PRINT D$" IS THIS OK ? Y/N"
 920 Z%=GET
 930 IF Z%>90 THEN Z%=Z%-32
 940 IF Z%<>78 AND Z%<>89 THEN 920
 950 IF Z%=78 THEN 900
 960 INPUT"DATE OF ANALYSIS "DATE$
 970 ENDPROC
 980:
 990 DEF PROCPLOT
1000 VDU26
1010 CLG
1020 CLS
1030 MULTF=650
1040 PRINT"IF YOU WANT TO PRINT YOUR CHROMATOGRAM YOU WILL NEED MDUMP"
1050 PRINT"DO TOU WANT TO PRINT YOUR CHROMATOGRAM? (Y/N?)"
1060 *FX15,1
1070 IF GET=89 PR%=1 ELSE PR%=0
1080 PRINT"TO PLOT THE CHROMATOGRAM ON SCALE THE DIGITAL VALUE OF THE INPUT IS M
FACTOR (MULTF)"
1090 PRINT
1100 PRINT"THE CURRENT VALUE OF MULTF =" MULTF
1110 PRINT
1120 PRINT"DO YOU WANT TO CHANGE THE VALUE OF MULTF? (Y/N?)";:INPUT Y2$
1130 IF Y2$="Y" OR Y2$="y" THEN 1140 ELSE 1150
```

```
1140 INPUT"ENTER NEW VALUE OF MULTF "MULTF
1150 CLS
1160 PLOT4,100,850
1170 PLOT5,100,100
1180 PLOT5,1100,100
1190 FOR I%=1 TO 1000
1200 PLOT4,100,100
1210 PLOT65,I%,DS(I%)*MULTF
1220 NEXT
1230PROCX(SP%(1)+100,(DS(SP%(1))*MULTF)+100)
1240PROCX(EP%(1)+100,(DS(EP%(1))*MULTF)+100)
1250 VDU28,0,3,39,0
1260 PRINT"DO YOU WANT TO REPLOT? (Y/N)"
1270 *FX15,1
1280 IF GET=89 THEN 1000 ELSE 1290
1290 ENDPROC
1300 DEF PROCALTER1
1310 P(1)=1:P(2)=2:P(3)=3:P(4)=4:P(5)=5
1320VDU28,0,4,39,0
1330FOR PEAK%=1 TO 5
1340 CLS
1350 *FX15,1
1360PRINT"RETENTION TIME - PEAK ";STR$PEAK%
1370 PRINT"A/S -INC.TIME   Z/X -DEC.TIME"
1380 PRINT"SPACE - NO FURTHER CHANGE"
1390 *FX15,1
1400PROCRT(PEAK%)
1410 IF F%<>1 THEN 1390
1420 *FX15,1
1430 CLS
1440PRINT"          ";"PEAK ";STR$PEAK%
1450 PRINT"A/S-INC.PEAK START  Z/X-DEC.PEAK START"
1460 PRINT"J/K-INC.PEAK END    N/M-DEC.PEAK END"
1470 PRINT"SPACE  -  NO FURTHER CHANGE"
1480 *FX15,1
1490PROCKEY3(PEAK%)
1500 IF F%<>1 THEN 1480
1510PROCBASE3(PEAK%)
1520 *FX15,1
1530 CLS
1540PRINT"DO YOU WANT TO ANALYSE A ";peak$(PEAK%+1);" PEAK? (Y/N?)"
1550 *FX15,1
1560 IF GET$="N" THEN P(PEAK%+1)=0
1570 IF P(PEAK%+1)=0 THEN PEAK%=5
1580NEXT
1590ENDPROC
1600:
1610 DEF PROCLINREG
1620 A=0 : B=0 : BARX=0 : BARY=0 : R=0
1630 SUMXY=0.0 : XSUM=0.0 : YSUM=0.0
1640 X2SUM=0.0 : Y2SUM=0.0
1650 FOR I%=1 TO N%
1660 SUMXY=SUMXY+STX(I%)*STY(I%)
1670 XSUM=XSUM+STX(I%)
1680 YSUM=YSUM+STY(I%)
1690 X2SUM=X2SUM+STX(I%)^2
1700 Y2SUM=Y2SUM+STY(I%)^2
```

```
1710 NEXT
1720  A=(SUMXY-XSUM*YSUM/N%)/(X2SUM-XSUM^2/N%)
1730 BARX=XSUM/N%
1740 BARY=YSUM/N%
1750 B=BARY-A*BARX
1760 R=(N%*SUMXY-XSUM*YSUM)/(SQR((N%*X2SUM-XSUM^2)*(N%*Y2SUM-YSUM^2)))
1770 PRINT "SLOPE= "A '"INTERCEPT= "B '"CORR.COEFF= "R
1780 ENDPROC
1790 DEF PROCCF
1800 CLS
1810 PRINT"CURRENT SLOPE OF CALIB. = "A
1820 PRINT"CURRENT Y-INTERCEPT = "B
1830 PRINT"CURRENT VOID VOL. TIME = "TN
1840 PRINT"CURRENT TOTAL VOL. COLUMN = "\T
1850 PRINT"CURRENT FLOW RATE = "SP" ml/min"
1860 PRINT"DO YOU WISH TO ENTER CALIB VALUES ? Y/N "
1870 Z%=GET
1880 IF Z%>90 THEN Z%=Z%-32
1890 IF Z%<>78 AND Z%<>89 THEN 1870
1900 IF Z%=78 THEN 2000
1910 INPUT"ENTER VOID VOL. TIME (min) "TN
1920 INPUT"TOTAL VOLUME OF COLUMN (ml) "\T
1930 INPUT"ENTER SLOPE OF CALIB "A
1940 INPUT"ENTER Y-INTERCEPT "B
1950 INPUT"ENTER FLOW RATE (ml/min) "SP
1960 PRINT"THE CURRENT CHROMATOGRAPHIC RUN TIME IS "DT" mins"
1970 PRINT"DO YOU WISH TO ENTER A NEW RUN TIME? (Y/N?)"
1980 ZZ%=GET
1990 IF ZZ%="Y" THEN 2000 ELSE 2010
2000 INPUT"ENTER NEW RUN TIME (mins) "DT
2010 ENDPROC
2020 DEF PROCX(X%,Y%)
2030 PLOT4,X%+25,Y%
2040 PLOT6,X%-25,Y%
2050 PLOT4,X%,Y%+25
2060 PLOT6,X%,Y%-25
2070 ENDPROC
2080:
2090 DEF PROCPRINT
2100PRINT "FILE:-  "FILE$
2110 PRINT"DATE OF ANALYSIS :- "DATE$
2120 PRINT"SAMPLE:-  "D$
2130PRINT"EXPTL.CODE:-  "EC$
2140 PRINT"FLOW:- "SP
2150 PRINT"DATA COLL TIME:-  "DT" min"
2160 ENDPROC
2170:
2180 DEF PROCRETRIEVE
2190INPUT"FILENAME ",FILE$
2200X%=OPENIN FILE$
2210 FOR I%=1 TO 1000
2220 INPUT£X%,I%,DS(I%)
2230 PRINT I%;"       ";DS(I%)
2240 NEXT
2250 CLOSE£X%
2260 ENDPROC
2270:
```

```
2280 DEF PROCANAL
2290 CLS
2300PRINT"OPTIONS AVAILABLE AT THIS STAGE"
2310 PRINT
2320PRINT
2330 PRINT"A - UP TO FIVE WELL-SEPARATED PEAKS: AUC ONLY"
2340 PRINT
2350 PRINT"B - UP TO FIVE WELL-SEPARATED PEAKS: AUC AND MW"
2360 PRINT
2370 PRINT"C - UP TO SIX OVERLAPPING PEAKS: AUC ONLY"
2380 PRINT
2390 PRINT"D - UP TO SIX OVERLAPPING PEAKS: AUC AND MW"
2400 *FX15,1
2410 K$=GET$
2420IF K$="A" THEN PROCanal(1)
2430IF K$="B" THEN PROCanal(2)
2440IF K$="C" THEN PROCanal(3)
2450IF K$="D" THEN PROCanal(4)
2460 ENDPROC
2470:
2480DEF PROCanal(N%)
2490 CLS
2500 PROCPLOT
2510 IF N%=1 OR N%=2 PROCALTER1 ELSE PROCALTER2
2520 IF PR%=0 THEN 2550
2530*MDUMP
2540 VDU3
2550 VDU26
2560 CLG
2570IF N%=1 PROCPROCESS1:PROCPRINT1
2580IF N%=2 PROCPROCESS2:PROCPRINT2
2590 IF N%=3 THEN PRCESS=3
2600IF N%=3 PROCPROCESS4:PROCPRINT4
2610 IF N%=4 THEN PRCESS=4
2620IF N%=4 PROCPROCESS4:PROCPRINT4
2630 ENDPROC
2640:
2650 DEF PROCKEY3(N%)


2660 *FX15,1
2670 F%=0
2680 IF INKEY(-99)=-1 THEN F%=1
2690 IF INKEY(-66)=-1 THEN F%=2
2700 IF INKEY(-98)=-1 THEN F%=3
2710 IF INKEY(-70)=-1 THEN F%=4
2720 IF INKEY(-86)=-1 THEN F%=5
2730 IF INKEY(-82)=-1 THEN F%=6
2740 IF INKEY(-67)=-1 THEN F%=7
2750 IF INKEY(-71)=-1 THEN F%=8
2760 IF INKEY(-102)=-1 THEN F%=9
2770 IF F%=0 THEN ENDPROC
2780 PROCX(SP%(N%)+100,(DS(SP%(N%))*MULTF)+100)
2790PROCX(EP%(N%)+100,(DS(EP%(N%))*MULTF)+100)
2800 IF F%=2 THENSP%(N%)=SP%(N%)+1
2810 IF F%=3 THENSP%(N%)=SP%(N%)-1
2820 IF F%=4 THENEP%(N%)=EP%(N%)+1
2830 IF F%=5 THENEP%(N%)=EP%(N%)-1
2840 IF F%=6 THENSP%(N%)=SP%(N%)+10
```

```
2850 IF F%=7 THENSP%(N%)=SP%(N%)-10
2860 IF F%=8 THENEP%(N%)=EP%(N%)+10
2870 IF F%=9 THENEP%(N%)=EP%(N%)-10
2880 IF SP%(N%)<1 THEN SP%(N%)=1
2890 IF EP%(N%)>1000 THEN EP%(N%)=1000
2900 PROCX(SP%(N%)+100,(DS(SP%(N%))*MULTF)+100)
2910PROCX(EP%(N%)+100,(DS(EP%(N%))*MULTF)+100)
2920 PRINT"START POINT = ";SP%(N%);"    END POINT = ";EP%(N%)
2930 PRINT"A/S-INC.PEAK START  Z/X-DEC.PEAK START"
2940 PRINT"J/K-INC.PEAK END    N/M-DEC.PEAK END"
2950 PRINT"SPACE  -  NO FURTHER CHANGE"
2960 ENDPROC
2970:
2980 DEF PROCBASE3(N%)
2990 PROCX(SP%(N%)+100,(DS(SP%(N%))*MULTF)+100)
3000 PROCX(EP%(N%)+100,(DS(EP%(N%))*MULTF)+100)
3010 PLOT4,SP%(N%)+100,(DS(SP%(N%))*MULTF)+100
3020 PLOT5,EP%(N%)+100,(DS(EP%(N%))*MULTF)+100
3030 ENDPROC
3040:
3050 DEF PROCPROCESS1
3060 F11=0:F12=0:F13=0:F14=0:F15=0
3070 FOR I%=SP%(1) TO EP%(1)
3080 CEXP1=DS(I%)-DS(SP%(1))
3090 CEXP1=CEXP1-(((DS(EP%(1))-DS(SP%(1)))/(EP%(1)-SP%(1)))*(I%-SP%(1)))
3100 F11=F11+CEXP1
3110 NEXT
3120 IF P(2)=0 THEN ENDPROC
3130 FOR I%=SP%(2) TO EP%(2)
3140 CEXP2=DS(I%)-DS(SP%(2))
3150 CEXP2=CEXP2-(((DS(EP%(2))-DS(SP%(2)))/(EP%(2)-SP%(2)))*(I%-SP%(2)))
3160 F12=F12+CEXP2
3170 NEXT
3180 IF P(3)=0 THEN ENDPROC
3190 FOR I%=SP%(3) TO EP%(3)
3200 CEXP3=DS(I%)-DS(SP%(3))
3210 CEXP3=CEXP3-(((DS(EP%(3))-DS(SP%(3)))/(EP%(3)-SP%(3)))*(I%-SP%(3)))
3220 F13=F13+CEXP3
3230 NEXT
3240 IF P(4)=0 THEN ENDPROC
3250 FOR I%=SP%(4) TO EP%(4)
3260 CEXP4=DS(I%)-DS(SP%(4))
3270 CEXP4=CEXP4-(((DS(EP%(4))-DS(SP%(4)))/(EP%(4)-SP%(4)))*(I%-SP%(4)))
3280 F14=F14+CEXP4
3290 NEXT
3300 IF P(5)=0 THEN ENDPROC
3310 FOR I%=SP%(5) TO EP%(5)
3320 CEXP5=DS(I%)-DS(SP%(5))
3330 CEXP5=CEXP5-(((DS(EP%(5))-DS(SP%(5)))/(EP%(5)-SP%(5)))*(I%-SP%(5)))
3340 F15=F15+CEXP5
3350 NEXT
3360 ENDPROC
3370:
3380 DEF PROCPRINT1
3390 PRINT"DO YOU WANT TO PRINT YOUR DATA ON PAPER? (Y/N?)"
3400 *FX15,1
3410 IF GET=89 THEN PR%=1 ELSE PR%=0
```

```
3420 CLS
3430 IF PR%=0 THEN 3450
3440 VDU2
3450 PROCPRINT
3460 PRINT
3470 PRINT
3480 PRINT
3490 PRINT"PEAK     RETN.TIME (min)   AUC"
3500 PRINT"---------------------------------------"
3510 PRINT
3520 @%=&20408
3530 PRINT" 1            ";DT*PH%(1)/1000,F11
3540 IF P(2)=0 THEN 3620
3550 PRINT" 2            ";DT*PH%(2)/1000,F12
3560 IF P(3)=0 THEN 3620
3570 PRINT" 3            ";DT*PH%(3)/1000,F13
3580 IF P(4)=0 THEN 3620
3590 PRINT" 4            ";DT*PH%(4)/1000,F14
3600 IF P(5)=0 THEN 3620
3610 PRINT" 5            ";DT*PH%(5)/1000,F15
3620 VDU3
3630 @%=10
3640 PRINT
3650 PRINT "PRESS SPACE BAR TO CONTINUE"
3660 *FX15,1
3670 L=GET
3680 IF L=20 THEN 3690
3690 ENDPROC
3700:
3710 DEF PROCRT(N%)
3720 *FX15,1
3730 F%=0
3740 IF INKEY(-99)=-1 THEN F%=1
3750 IF INKEY(-66)=-1 THEN F%=2
3760 IF INKEY(-98)=-1 THEN F%=3
3770 IF INKEY(-82)=-1 THEN F%=4
3780 IF INKEY(-67)=-1 THEN F%=5
3790 IF F%=0 THEN ENDPROC
3800 PROCX(PH%(N%)+100,(DS(PH%(N%))*MULTF)+100)
3810 IF F%=2 THEN PH%(N%)=PH%(N%)+1
3820 IF F%=3 THEN PH%(N%)=PH%(N%)-1
3830 IF F%=4 THEN PH%(N%)=PH%(N%)+10
3840 IF F%=5 THEN PH%(N%)=PH%(N%)-10
3850 PROCX(PH%(N%)+100,(DS(PH%(N%))*MULTF)+100)
3860 PRINT"RETENTION TIME - PEAK ";STR$PEAK%
3870 PRINT"A/S -INC.TIME   Z/X -DEC.TIME"
3880 PRINT"SPACE - NO FURTHER CHANGE"
3890 PRINT"       DATA POINT = ";PH%(N%)
3900 ENDPROC
3910:
3920 DEF PROCPROCESS2
3930 F11=0:F12=0:F13=0:F14=0:F15=0
3940 F0=0
3950 F21=0:F22=0:F23=0:F24=0:F25=0
3960 F31=0:F32=0:F33=0:F34=0:F35=0
3970 ST%=DT*6000/1000
3980 FOR I%=SP%(1) TO EP%(1)
```

```
3990 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
4000 CEXP1=DS(I%)-DS(SP%(1))
4010 CEXP1=CEXP1-(((DS(EP%(1))-DS(SP%(1)))/(EP%(1)-SP%(1)))*(I%-SP%(1)))
4020 F11=F11+CEXP1
4030 F21=F21+CEXP1*F0
4040 F31=F31+CEXP1/F0
4050 NEXT
4060 IF P(2)=0 THEN ENDPROC
4070 FOR I%=SP%(2) TO EP%(2)
4080 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
4090 CEXP2=DS(I%)-DS(SP%(2))
4100 CEXP2=CEXP2-(((DS(EP%(2))-DS(SP%(2)))/(EP%(2)-SP%(2)))*(I%-SP%(2)))
4110 F12=F12+CEXP2
4120 F22=F22+CEXP2*F0
4130 F32=F32+CEXP2/F0
4140 NEXT
4150 IF P(3)=0 THEN ENDPROC
4160 FOR I%=SP%(3) TO EP%(3)
4170 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
4180 CEXP3=DS(I%)-DS(SP%(3))
4190 CEXP3=CEXP3-(((DS(EP%(3))-DS(SP%(3)))/(EP%(3)-SP%(3)))*(I%-SP%(3)))
4200 F13=F13+CEXP3
4210 F23=F23+CEXP3*F0
4220 F33=F33+CEXP3/F0
4230 NEXT
4240 IF P(4)=0 THEN ENDPROC
4250 FOR I%=SP%(4) TO EP%(4)
4260 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
4270 CEXP4=DS(I%)-DS(SP%(4))
4280 CEXP4=CEXP4-(((DS(EP%(4))-DS(SP%(4)))/(EP%(4)-SP%(4)))*(I%-SP%(4)))
4290 F14=F14+CEXP4
4300 F24=F24+CEXP4*F0
4310 F34=F34+CEXP4/F0
4320 NEXT
4330 IF P(5)=0 THEN ENDPROC
4340 FOR I%=SP%(5) TO EP%(5)
4350 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
4360 CEXP5=DS(I%)-DS(SP%(5))
4370 CEXP5=CEXP5-(((DS(EP%(5))-DS(SP%(5)))/(EP%(5)-SP%(5)))*(I%-SP%(5)))
4380 F15=F15+CEXP5
4390 F25=F25+CEXP5*F0
4400 F35=F35+CEXP5/F0
4410 NEXT
4420 ENDPROC
4430:
4440 DEF PROCPRINT2
4450 PRINT"DO YOU WANT TO PRINT YOUR DATA ON PAPER? (Y/N?)"
4460 *FX15,1
4470 IF GET=89 THEN PR%=1 ELSE PR%=0
4480 CLS
4490 IF PR%=0 THEN 4510
4500 VDU2
4510 PROCPRINT
4520 PRINT
4530 PRINT
4540 PRINT
4550 PRINT"PEAK     RETN.TIME (min)    AUC"
```

```
4560 PRINT"---------------------------------------"
4570 PRINT
4580 @%=&20408
4590 PRINT" 1            ";DT*PH%(1)/1000,F11
4600 IF P(2)=0 THEN 4680
4610 PRINT" 2            ";DT*PH%(2)/1000,F12
4620 IF P(3)=0 THEN 4680
4630 PRINT" 3            ";DT*PH%(3)/1000,F13
4640 IF P(4)=0 THEN 4680
4650 PRINT" 4            ";DT*PH%(4)/1000,F14
4660 IF P(5)=0 THEN 4680
4670 PRINT" 5            ";DT*PH%(5)/1000,F15
4680 PRINT
4690 PRINT
4700 PRINT
4710 @%=&10507
4720 PRINT"PEAK   NUM.AVE.MW   WT.AVE.MW   Mw/Mn"
4730 PRINT"---------------------------------------"
4740 PRINT
4750 PRINT" 1      ";F11/F31,F21/F11,(F21*F31/(F11^2))
4760 IF P(2)=0 THEN 4840
4770 PRINT" 2      ";F12/F32,F22/F12,(F22*F32/(F12^2))
4780 IF P(3)=0 THEN 4840
4790 PRINT" 3      ";F13/F33,F23/F13,(F23*F33/(F13^2))
4800 IF P(4)=0 THEN 4840
4810 PRINT" 4      ";F14/F34,F24/F14,(F24*F34/(F14^2))
4820 IF P(5)=0 THEN 4840
4830 PRINT" 5      ";F15/F35,F25/F15,(F25*F35/(F15^2))
4840 VDU3
4850 @%=10
4860 PRINT
4870 PRINT"PRESS SPACE BAR TO CONTINUE"
4880 *FX15,1
4890 L=GET
4900 IF L=20 THEN 4910
4910 ENDPROC
4920:
4930 DEF PROCALTER2
4940 P(1)=1:P(2)=2:P(3)=3:P(4)=4:P(5)=5:P(6)=6
4950 VDU28,0,4,39,0
4960 CLS
4970 *FX15,1
4980 PRINT"BASELINE OF WHOLE CHROMATOGRAM"
4990 PRINT"A/S-INC.PEAK START  Z/X-DEC.PEAK START"
5000 PRINT"J/K-INC.PEAK END    N/M-DEC.PEAK END"
5010 PRINT"SPACE  -  NO FURTHER CHANGE"
5020 *FX15,1
5030 PROCKEY3(6)
5040 IF F%<>1 THEN 5020
5050 PROCBASE3(6)
5060 *FX15,1
5070 CLS
5080 FOR C%=1 TO 6
5090 PRINT"MARK PEAK CUT ";STR$C%
5100 PRINT"A/S- INC. TIME    Z/X- DEC. TIME"
5110 PRINT"SPACE  -  NO FURTHER CHANGE"
5120 *FX15,1
```

```
5130 PROCCUT(C%)
5140IF F%<>1 THEN 5120
5150 *FX15,1
5160 PROCVERT(C%)
5170 *FX15,1
5180 CLS
5190 IF C%=6 THEN 5240
5200 PRINT"DO YOU WANT TO MAKE A ";cut$(C%+1);" CUT? (Y/N?)"
5210 *FX15,1
5220 IF GET$="N" THEN P(C%+1)=0
5230 IF P(C%+1)=0 THEN C%=6
5240 NEXT
5250 ENDPROC
5260:
5270 DEF PROCCUT(N%)
5280 *FX15,1
5290 F%=0
5300 IF INKEY(-99)=-1 THEN F%=1
5310 IF INKEY(-66)=-1 THEN F%=2
5320 IF INKEY(-98)=-1 THEN F%=3
5330 IF INKEY(-82)=-1 THEN F%=4
5340 IF INKEY(-67)=-1 THEN F%=5
5350 IF F%=0 THEN ENDPROC
5360 PROCX(CUT%(N%)+100,(DS(CUT%(N%))*MULTF)+100)
5370 IF F%=2 THEN CUT%(N%)=CUT%(N%)+1
5380 IF F%=3 THEN CUT%(N%)=CUT%(N%)-1
5390 IF F%=4 THEN CUT%(N%)=CUT%(N%)+10
5400 IF F%=5 THEN CUT%(N%)=CUT%(N%)-10
5410 PROCX(CUT%(N%)+100,(DS(CUT%(N%))*MULTF)+100)
5420 PRINT"MARK PEAK CUT ";STR$C%
5430 PRINT"A/S- INC. TIME    Z/X- DEC. TIME"
5440 PRINT"SPACE  -  NO FURTHER CHANGE"
5450 PRINT"       DATA POINT =";CUT%(N%)
5460 ENDPROC
5470:
5480 DEF PROCVERT(N%)
5490 PROCX(CUT%(N%)+100,(DS(CUT%(N%))*MULTF)+100)
5500 PLOT4,CUT%(N%)+100,(DS(CUT%(N%))*MULTF)+100
5510 PLOT5,CUT%(N%)+100,(DS(SP%(6))+(DS(EP%(6))-DS(SP%(6)))*(CUT%(N%)-SP%(6))/(EP%(6)-SP%(6
MULTF+100
5520 ENDPROC
5530:
5540 DEF PROCPROCESS4
5550 F11=0:F12=0:F13=0:F14=0:F15=0:F16=0:F17=0:F18=0:F19=0:F110=0:F111=0:F112=0:F113=0
5560 F21=0:F22=0:F23=0:F24=0:F25=0:F26=0:F27=0:F28=0:F29=0:F210=0:F211=0:F212=0:F213=0
5570 F31=0:F32=0:F33=0:F34=0:F35=0:F36=0:F37=0:F38=0:F39=0:F310=0:F311=0:F312=0:F313=0
5580 F0=0
5590 ST%=DT*6000/1000
5600 FOR I%=SP%(6) TO EP%(6)
5610 IF PRCESS=3 THEN 5630 ELSE 5620
5620 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
5630 CEXP1=DS(I%)-DS(SP%(6))
5640 CEXP1=CEXP1-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
5650 F11=F11+CEXP1
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```
5660 IF PRCESS=3 THEN 5690 ELSE 5670
5670 F21=F21+CEXP1*F0
5680 F31=F31+CEXP1/F0
5690 NEXT
5700 FOR I%=SP%(6) TO CUT%(1)
5710 IF PRCESS=3 THEN 5730 ELSE 5720
5720 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
5730 CEXP2=DS(I%)-DS(SP%(6))
5740 CEXP2=CEXP2-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
5750 F12=F12+CEXP2
5760 IF PRCESS=3 THEN 5790 ELSE 5770
5770 F22=F22+CEXP2*F0
5780 F32=F32+CEXP2/F0
5790 NEXT
5800 IF P(2)=0 THEN 5810 ELSE 5920
5810 FOR I%=(CUT%(1)+1) TO EP%(6)
5820 IF PRCESS=3 THEN 5840 ELSE 5830
5830 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
5840 CEXP7=DS(I%)-DS(SP%(6))
5850 CEXP7=CEXP7-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
5860 F17=F17+CEXP7
5870 IF PRCESS=3 THEN 5900 ELSE 5880
5880 F27=F27+CEXP7*F0
5890 F37=F37+CEXP7/F0
5900 NEXT
5910 IF P(2)=0 THEN ENDPROC
5920 FOR I%=(CUT%(1)+1) TO CUT%(2)
5930 IF PRCESS=3 THEN 5950 ELSE 5940
5940 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
5950 CEXP3=DS(I%)-DS(SP%(6))
5960 CEXP3=CEXP3-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
5970 F13=F13+CEXP3
5980 IF PRCESS=3 THEN 6010 ELSE 5990
5990 F23=F23+CEXP3*F0
6000 F33=F33+CEXP3/F0
6010 NEXT
6020 IF P(3)=0 THEN 6030 ELSE 6140
6030 FOR I%=(CUT%(2)+1) TO EP%(6)
6040 IF PRCESS=3 THEN 6060 ELSE 6050
6050 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
6060 CEXP8=DS(I%)-DS(SP%(6))
6070 CEXP8=CEXP8-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
6080 F18=F18+CEXP8
6090 IF PRCESS=3 THEN 6120 ELSE 6100
6100 F28=F28+CEXP8*F0
6110 F38=F38+CEXP8/F0
6120 NEXT
6130 IF P(3)=0 THEN ENDPROC
6140 FOR I%=(CUT%(2)+1) TO CUT%(3)
6150 IF PRCESS=3 THEN 6170 ELSE 6160
6160 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
6170 CEXP4=DS(I%)-DS(SP%(6))
6180 CEXP4=CEXP4-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
6190 F14=F14+CEXP4
6200 IF PRCESS=3 THEN 6230 ELSE 6210
6210 F24=F24+CEXP4*F0
6220 F34=F34+CEXP4/F0
```

```
6230 NEXT
6240 IF P(4)=0 THEN 6250 ELSE 6360
6250 FOR I%=(CUT%(3)+1) TO EP%(6)
6260 IF PRCESS=3 THEN 6280 ELSE 6270
6270 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
6280 CEXP9=DS(I%)-DS(SP%(6))
6290 CEXP9=CEXP9-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
6300 F19=F19+CEXP9
6310 IF PRCESS=3 THEN 6340 ELSE 6320
6320 F29=F29+CEXP9*F0
6330 F39=F39+CEXP9/F0
6340 NEXT
6350 IF P(4)=0 THEN ENDPROC
6360 FOR I%=(CUT%(3)+1) TO CUT%(4)
6370 IF PRCESS=3 THEN 6390 ELSE 6380
6380 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
6390 CEXP5=DS(I%)-DS(SP%(6))
6400 CEXP5=CEXP5-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
6410 F15=F15+CEXP5
6420 IF PRCESS=3 THEN 6450 ELSE 6430
6430 F25=F25+CEXP5*F0
6440 F35=F35+CEXP5/F0
6450 NEXT
6460 IF P(5)=0 THEN 6470 ELSE 6580
6470 FOR I%=(CUT%(4)+1) TO EP%(6)
6480 IF PRCESS=3 THEN 6500 ELSE 6490
6490 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
6500 CEXP10=DS(I%)-DS(SP%(6))
6510 CEXP10=CEXP10-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
6520 F110=F110+CEXP10
6530 IF PRCESS=3 THEN 6560 ELSE 6540
6540 F210=F210+CEXP10*F0
6550 F310=F310+CEXP10/F0
6560 NEXT
6570 IF P(5)=0 THEN ENDPROC
6580 FOR I%=(CUT%(4)+1) TO CUT%(5)
6590 IF PRCESS=3 THEN 6610 ELSE 6600
6600 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
6610 CEXP6=DS(I%)-DS(SP%(6))
6620 CEXP6=CEXP6-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
6630 F16=F16+CEXP6
6640 IF PRCESS=3 THEN 6670 ELSE 6650
6650 F26=F26+CEXP6*F0
6660 F36=F36+CEXP6/F0
6670 NEXT
6680 FOR I%=(CUT%(5)+1) TO EP%(6)
6690 IF PRCESS=3 THEN 6710 ELSE 6700
6700 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
6710 CEXP11=DS(I%)-DS(SP%(6))
6720 CEXP11=CEXP11-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
6730 F111=F111+CEXP11
6740 IF PRCESS=3 THEN 6770 ELSE 6750
6750 F211=F211+CEXP11*F0
6760 F311=F311+CEXP11/F0
6770 NEXT
6780 IF P(6)=0 THEN ENDPROC
6790 FOR I%=(CUT%(5)+1) TO CUT%(6)
```

```
6800 IF PRCESS=3 THEN 6820 ELSE 6810
6810 F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
6820 CEXP12=DS(I%)-DS(SP%(6))
6830 CEXP12=CEXP12-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
6840 F112=F112+CEXP12
6850 IF PRCESS=3 THEN 6880 ELSE 6860
6860 F212=F212+CEXP12*F0
6870 F312=F312+CEXP12/F0
6880 NEXT
6890 FOR I%=(CUT%(6)+1) TO EP%(6)
6900 IF PRCESS=3 THEN 6920 ELSE 6910
6910  F0=10^(A*((ST%*I%/6000)*SP-TN*SP)/(VT-TN*SP)+B)
6920 CEXP13=DS(I%)-DS(SP%(6))
6930 CEXP13=CEXP13-(((DS(EP%(6))-DS(SP%(6)))/(EP%(6)-SP%(6)))*(I%-SP%(6)))
6940 F113=F113+CEXP13
6950 IF PRCESS=3 THEN 6980 ELSE 6960
6960 F213=F213+CEXP13*F0
6970 F313=F313+CEXP13/F0
6980 NEXT
6990 ENDPROC
7000:
7010:
7020 DEF PROCPRINT4
7030 PRINT"DO YOU WANT TO PRINT YOUR DATA? (Y/N?)"
7040 *FX15,1
7050 IF GET=89 THEN PR%=1 ELSE PR%=0
7060 CLS
7070 IF PR%=0 THEN 7090
7080 VDU2
7090 PROCPRINT
7100 PRINT
7110 PRINT
7120 PRINT
7130 PRINT "TOTAL AREA UNDER CHROMATOGRAM = ";F11
7140PRINT
7150PRINT
7160PRINT"PEAK CUT       AREA OF CUT       % OF TOTAL AREA"
7170PRINT"-------------------------------------------------"
7180PRINT
7190 @%=&20408
7200PRINT"   1             ";F12,;"          ";F12*100/F11
7210 IF P(2)=0 THEN 7220 ELSE 7240
7220PRINT"   2             ";F17,;"          ";F17*100/F11
7230 IF P(2)=0 THEN 7430
7240PRINT"   2             ";F13,;"          ";F13*100/F11
7250 IF P(3)=0 THEN 7260 ELSE 7280
7260PRINT"   3             ";F18,;"          ";F18*100/F11
7270 IF P(3)=0 THEN 7430
7280PRINT"   3             ";F14,;"          ";F14*100/F11
7290 IF P(4)=0 THEN 7300 ELSE 7320
7300PRINT"   4             ";F19,;"          ";F19*100/F11
7310 IF P(4)=0 THEN 7430
7320PRINT"   4             ";F15,;"          ";F15*100/F11
7330 IF P(5)=0 THEN 7340 ELSE 7360
7340PRINT"   5             ";F110,;"          ";F110*100/F11
7350 IF P(5)=0 THEN 7430
7360PRINT"   5             ";F16,;"          ";F16*100/F11
```

```
7370 IF P(6)=0 THEN 7380 ELSE 7400
7380PRINT"   6            ";F111,;"          ";F111*100/F11
7390 IF P(6)=0 THEN 7430
7400 PRINT"   6            ";F112,;"          ";F112*100/F11
7410PRINT"   7            ";F113,;"          ";F113*100/F11
7420 IF PRCESS=3 THEN 7720
7430PRINT
7440PRINT
7450PRINT
7460@%=&10507
7470PRINT"PEAK CUT    NUM.AVE.MW     WT.AVE.MW        Mw/Mn"
7480PRINT"-------------------------------------------------"
7490PRINT
7500PRINT"  1          ";F12/F32,F22/F12,(F22*F32/(F12^2))
7510 IF P(2)=0 THEN 7520 ELSE 7540
7520PRINT"  2          ";F17/F37,F27/F17,(F27*F37/(F17^2))
7530 IF P(2)=0 THEN 7720
7540PRINT"  2          ";F13/F33,F23/F13,(F23*F33/(F13^2))
7550 IF P(3)=0 THEN 7560 ELSE 7580
7560PRINT"  3          ";F18/F38,F28/F18,(F28*F38/(F18^2))
7570 IF P(3)=0 THEN 7720
7580PRINT"  3          ";F14/F34,F24/F14,(F24*F34/(F14^2))
7590 IF P(4)=0 THEN 7600 ELSE 7620
7600PRINT"  4          ";F19/F39,F29/F19,(F29*F39/(F19^2))
7610 IF P(4)=0 THEN 7720
7620PRINT"  4          ";F15/F35,F25/F15,(F25*F35/(F15^2))
7630 IF P(5)=0 THEN 7640 ELSE 7660
7640PRINT"  5          ";F110/F310,F210/F110,(F210*F310/(F110^2))
7650 IF P(5)=0 THEN 7720
7660PRINT"  5          ";F16/F36,F26/F16,(F26*F36/(F16^2))
7670 IF P(6)=0 THEN 7680 ELSE 7700
7680PRINT"  6          ";F111/F311,F211/F111,(F211*F311/(F111^2))
7690 IF P(6)=0 THEN 7720
7700PRINT"  6          ";F112/F312,F212/F112,(F212*F312/(F112^2))
7710PRINT"  7          ";F113/F313,F213/F113,(F213*F313/(F113^2))
7720 VDU3
7730 @%=10
7740 PRINT
7750 PRINT"PRESS SPACE BAR TO CONTINUE"
7760 *FX15,1
7770 L=GET
7780 IF L=20 THEN 4910
7790 ENDPROC
7800:
```

APPENDIX A8: TABLES OF EXPERIMENTAL DATA

Table A8.2.1:
The effect of reaction time on the MCR
of MTX-HSA conjugates (ambient temperature, PBS, pH 7.45)
HSA Concentration = 10 mg/ml

Time (Hours)	Mean MCR (±sd) [n = 3]
1.2	1.22 (±0.18)
2.3	1.97 (±0.10)
4.0	2.33 (±0.14)
24.0	3.12 (±0.18)

Table A8.2.2:
The effect of reaction time on the MCR
of MTX-HSA conjugates (25°C, PBS, pH 7.35)
HSA Concentration = 10 mg/ml

Time (Hours)	Mean MCR (±sd) [n = 2]
1.0	2.33 (±0.05)
2.0	2.74 (±0.06)
4.0	4.22 (±0.08)
9.3	4.62 (±0.03)
20.0	5.06 (±0.03)
23.0	5.11

Table A8.2.3:
The effect of the molar ratio of MTX on the MCR
of MTX-HSA conjugates (25°C, PBS, pH 7.35)
HSA Concentration = 10 mg/ml

Molar Ratio HSA:MTX:ECDI	Mean MCR (±sd) [n]
1:14.6:43.2	1.81 (±0.04) [2]
1:29.2:43.2	2.88 (±0.29) [6]
1:58.3:43.2	5.08 (±0.03) [3]
1:116.5:43.2	7.88 (±0.41) [3]

Table A8.2.4:
The effect of the molar ratio of ECDI on the MCR of MTX-HSA conjugates (25°C, PBS, pH 7.35)
HSA Concentration = 10 mg/ml

Molar Ratio HSA:MTX:ECDI	Mean MCR (±sd) [n]
1:116.6:0	0.01 [1]
1:58.3:21.6	2.90 (±0.08) [2]
1:58.3:43.2	5.08 (±0.03) [3]
1:58.3:86.5	8.62 (±0.14) [2]
1:58.3:173.0	13.59 (±0.07) [2]

Table A8.2.5:
The effect of increasing the molar ratio of MTX and ECDI (1:1.5) relative to HSA on the MCR of MTX-HSA conjugates (25°C, PBS pH 7.35)
HSA Concentration = 10 mg/ml

Molar Ratio HSA:MTX:ECDI	Mean MCR (±sd) [n]
1:29.2:43.2	2.88 (±0.29) [6]
1:58.3:86.5	8.62 (±0.14) [2]
1:87.5:129.7	14.39 (±0.21) [2]
1:116.5:173.0	19.43 (±0.44) [4]
1:145.8:216.2	23.55 (±0.44) [2]

Table A8.2.6:
The effect of pH on the MCR of MTX-HSA conjugates (HSA:MTX:ECDI = 1:58.3:86.4, 25°C)
HSA Concentration = 10 mg/ml

Measured pH	Mean MCR (±sd) [n = 2]
6.03	10.42 (±0.04)
6.23	9.84 (±0.05)
6.40	9.42 (±0.10)
6.56	9.06 (±0.09)
6.70	9.05 (±0.10)
6.91	8.70 (±0.14)
7.35	8.62 (±0.14)
8.05	8.74 (±0.07)

Table A8.2.7:
Conjugation of MTX to HSA via the active ester of MTX
(DMF/PBS pH 7.4)
HSA Concentration = 10 mg/ml

Volume of active ester solution ml	Approximate ratio of active MTX:HSA in reaction mix	MCR after 4.5-5.5 hours	MCR over-night (24-30 hours)
0.01	0.6	0.69	0.83
0.04	2.3	1.86	2.01
0.08	4.6	4.03	3.81
0.16	9.2	8.12	7.33
0.32	18.4	13.44	13.55
0.64	36.7	21.85*	22.72

* some precipitation

Table A8.2.8:
Release of free MTX from MTX-HSA conjugates of different MCR
(37°C, PBS, pH 7.4)
HSA Concentration = 7.7×10^{-6}M

Mean MCR = 4.7 (±0.9)		Mean MCR = 19.5 (±0.8)	
Time Hours	Mean % free MTX (± sd)	Time Hours	Mean % free MTX (± sd)
0.3	0.13 (±0.13)	0.3	0.05 -
1.6	0.42 (±0.01)	1.3	0.33 (±0.04)
2.6	0.83 (±0.10)	2.4	0.67 -
-	-	3.4	0.98 (±0.04)
4.1	1.12 (±0.18)	4.5	1.26 (±0.09)
5.4	1.39 (±0.09)	5.6	1.56 (±0.13)
6.6	1.64 (±0.07)	6.7	1.78 (±0.13)
7.9	1.89 (±0.05)	7.7	1.87 (±0.15)
9.1	2.12 (±0.07)	8.8	2.12 (±0.06)
10.3	2.30 (±0.09)	9.9	2.27 -
11.6	2.50 (±0.06)	10.9	2.40 -
12.9	2.69 (±0.09)	11.9	2.49 (±0.02)
14.1	2.88 (±0.11)	-	-
15.3	3.06 (±0.13)	-	-
16.7	3.24 -	18.1	3.09 (±0.10)
20.4	3.41 -	19.4	3.18 (±0.07)
25.1	3.85 (±0.39)	24.0	3.63 (±0.13)

Table A8.2.9:
Release of free MTX from MTX-HSA conjugates with different conjugation reaction times (37°C, PBS, pH 7.4)

Run Number	mean of 1 and 2		mean of 3 and 4		5	
Ratio HSA:MTX:ECDI	1:58.3:43.2		1:116.5:43.2		1:116.5:43.2	
Reaction Time	24 hours		4 hours		25 hours	
Mean MCR	4.7 (±0.9)		5.3 (±1.2)		4.8	
MTX concentration	3.6×10^{-5}M		4.02×10^{-5}M		3.6×10^{-5}M	
	Time Hours	Mean % free MTX (±sd)	Time Hours	Mean % free MTX (±sd)	Time Hours	Mean % free MTX
	0.3	0.13 (±0.13)	0.2	0.27 (±0.07)	0.2	0.03
	1.6	0.42 (±0.01)	1.3	1.63 (±0.13)	1.3	0.42
	2.6	0.83 (±0.10)	2.3	2.59 (±0.10)	2.3	0.66
	4.1	1.12 (±0.18)	4.3	3.84 (±0.19)	4.4	1.27
	6.6	1.64 (±0.07)	6.3	4.70 (±0.25)	6.5	1.72
	7.9	1.89 (±0.05)	8.4	5.33 (±0.23)	7.6	1.93
	10.3	2.30 (±0.09)	10.5	5.74 (±0.15)	10.6	2.45
	12.9	2.69 (±0.09)	12.6	6.15 (±0.40)	12.7	2.78
	15.3	3.06 (±0.13)	16.0	6.53 (±0.30)	18.0	3.20
	25.1	3.85 (±0.39)	25.1	7.18 (±0.16)	25.0	3.94

Table A8.2.10:
Release of free MTX from MTX-HSA conjugates: effect of refractionating. (37°C, PBS, pH 7.4)

Fractionation Run	A		A refractionated		B		B refractionated	
HSA concentration	7.72×10^{-6}M		3.96×10^{-7}M		7.72×10^{-6}M		1.98×10^{-6}M	
MTX concentration	1.46×10^{-4}M		6.77×10^{-6}M		1.55×10^{-4}M		3.74×10^{-5}M	
MCR	18.91		17.10		20.08		18.89	
	Time Hours	% free MTX	Time Hours	% free MTX	Time Hours	% free MTX	Time Hours	% free MTX
	0.3	0.05	0.3	0.00	0.3	0.05	0.3	0.06
	1.3	0.30	1.3	0.11	1.3	0.35	1.3	0.12
	2.4	0.67	2.3	0.19	2.4	0.67	2.5	0.21
	4.5	1.20	-	-	4.8	1.32	4.6	0.28
	7.7	1.88	8.2	0.37	8.2	2.05	-	-
	11.9	2.50	11.8	0.48	11.7	2.47	12.2	0.51
	18.1	3.16	17.5	0.66	18.3	3.02	-	-
	24.0	3.72	24.6	0.84	23.2	3.45	-	-
	-	-	-	-	35.9	4.47	-	-
	-	-	-	-	47.6	4.91	-	-
	65.0	5.00	66.0	1.71	64.8	5.10	-	-

Table A8.2.11:
Effect of pH on the release of free MTX (%) from MTX-HSA conjugates
HSA Concentration 6.3×10^{-6}M

Nominal pH	5	6	7	8	9	10	12
Measured pH	5.24	6.14	7.07	8.10	8.98	10.09	12.03
Buffer Type	——Citric Acid/phosphate——				——Boric Acid/NaOH——		
Time (Hours)							
Initial	0.66	0.49	0.41	0.37	0.33	4.09	8.27
2	0.74	0.66	0.74	1.23	1.72	4.59	8.19
4	1.15	1.10	1.43	1.72	2.46	5.32	7.94
8	1.47	1.97	1.88	2.54	3.44	6.55	8.19
24	2.54	3.11	3.93	4.34	5.32	7.61	8.19

Table A8.2.12:
Release of free MTX from MTX-HSA (ester conjugated)
(37°C, PBS, pH 7.4)

Sample No	1		2	
Reaction Time	5.5 hours		5.5 hours	
MTX Conc	13.72×10^{-5}M		12.30×10^{-5}M	
MCR	21.85		20.37	
	Time (Hours)	% free MTX	Time (Hours)	% free MTX
	Initial	-	Initial	0.03
	1.1	0.19	1.2	0.10
	2.3	0.30	2.4	0.34
	3.5	0.41	3.6	0.46
	4.7	0.50	4.8	0.57
	5.9	0.59	6.0	0.65
	7.1	0.67	7.2	0.73
	8.3	0.74	8.4	0.83
	9.5	0.78	9.6	0.89
	10.7	0.84	10.8	0.99
	11.9	0.90	44.6	1.87
	-	-	60.5	2.10

Table A8.3.1:
The effect of trypsin on the release of free MTX from MTX-HSA conjugates (37°C, PBS, pH 7.4)

Sample	With Trypsin		Without Trypsin	
No of runs	2		4	
Mean HSA conc	7.68 (±0.09) x 10^{-6}M		7.74 (±0.01) x 10^{-6}M	
Mean MCR	21.3 (±0.5)		20.5 (±1.3)	
Mean Try:HSA (molar ratio)	0.28 (±0.01)		-	
	Time (Hours)	% free MTX (±sd)	Time (Hours)	% free MTX (±sd)
	0.5	0.07 (±0.01)	0.3	0.06 (±0.03)
	1.8	0.66 (±0.18)	1.4	0.42 (±0.15)
	4.3	1.53 (±0.12)	4.2	1.29 (±0.16)
	8.1	2.35 (±0.16)	7.7	2.17 (±0.33)
	11.9	3.02 -	11.9	2.76 (±0.38)
	17.6	3.74 -	18.1	3.13 (±0.17)
	24.0	3.87 (±0.28)	24.0	3.64 (±0.09)

Table A8.3.2:
The effect of different levels of trypsin on the release of free MTX from MTX-HSA conjugates (37°C, PBS, pH 7.4)

Sample A 2.82*		Sample B 0.29*		Sample C 0.03*		NT 0.00*	
Time (Hours)	% free MTX	Time (Hours)	% free MTX	Time (Hours)	% free MTX	Time (Hours)	% free MTX
-	-	-	-	-	-	0.2	0.01
1.6	-	2.7	0.62	4.0	1.12	5.2	0.89
7.4	1.79	-	-	-	-	-	-
-	-	21.0	2.84	22.3	2.94	23.5	3.04
-	-	30.1	3.62	31.2	3.37	32.3	3.62
42.0	4.29	43.1	4.09	44.2	3.94	45.2	4.34
51.9	4.71	53.1	4.96	54.3	4.91	-	-

* = molar ration of trypsin to HSA
NT = no trypsin
HSA Concentration Sample A = 7.75×10^{-6}M
Sample B = 7.45×10^{-6}M
Sample C = 7.72×10^{-6}M
NT = 7.75×10^{-6}M

Table A8.3.3:
The effect of trypsin on the molecular weight distribution of human serum albumin (37°C, PBS, pH 7.4)

	Molecular weight fractions (Mw x 10^3)						
Sample Code	HSA dimer 140-120	HSA monomer 65-60	40	31-27	21-15	12-9	<1.5
A-0H (S)	4.1	75.6	-	13.2	-	-	-
A-20H (S)	2.1	23.3	3.3	17.8	16.1	13.5	5.4
B-0H (S)	5.7	82.9	-	6.2	-	-	-
B-20H (S)	1.9	43.9	3.5	13.0	9.4	6.7	3.9
C-0H	10.2	85.0	-	-	-	-	-
C-20H	8.6	86.3	-	3.6	-	-	-

Results are expressed as % total area

A = Trypsin:HSA molar ratio = 2.82:1
B = Trypsin:HSA molar ratio = 0.29:1
C = HSA

0H = initial
20H = 20 hours at 37°C (S) = subtracted chromatogram

Table A8.3.4:
The molecular weight distribution of MTX-HSA conjugates in the presence and absence of trypsin in buffer solutions (pH 7.4 and 5.6) at 37°C

	Initial	24 Hour						
MW[1] Sample	59,000	57,000	52,000	23,000	9,000	3,000	Free MTX[2]	<1500
pH 7.4	98.8 (±0.3)	94.6 (±1.5)	-	-	-	-	3.4 (±0.4)	-
pH 5.6	98.3 (±0.2)	96.5 (±0.6)	-	-	-	-	1.0 (±0.2)	
pH 7.4 + Trypsin	94.4 (±1.9)	-	33.6 (±1.6)	23.7 (±1.1)	16.1 (±0.8)	10.7 (±0.6)	5.5 (±0.2)	16.7 (±1.3)
pH 5.6 + Trypsin	97.2 (±0.4)	-	60.6 (±2.8)	21.1 (±1.0)	9.1 (±1.2)	6.5 (±0.7)	1.6 (±0.6)	1.7 (±0.4)

(±sd)

Results expressed as % of total area

1 = MW - molecular weight
2 = expressed as % of total calculated from standard peak height
HSA Concentration = 4.2×10^{-6}M

Table A8.4.1:The molecular weight distribution of a MTX-L-copolymer conjugate incubated with trypsin in buffer (PBS, pH 7.4) at 37°C

Trypsin:copolymer molar ratio = 1:10
Coploymer Concentration = 4.7×10^{-6}M

	Molecular weight x 10^3							
Time at 37°C/hours	>60	46	30	15	8	4	1.8	<1.5
0	17.7	33.4	-	28.6	-	7.3	3.7	9.4
2	-	-	22.0	8.6	13.9	17.9	6.4	31.1
6	-	-	13.5	12.6	13.9	19.7	5.9	34.6
16	-	-	11.2	11.0	14.5	20.3	5.3	37.8
24	-	-	10.6	11.4	13.8	20.8	5.4	38.1

results expressed as % of total area